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A multidisciplinary approach to the comparison of three contrasting treatments on both lampenflora community and underlying rock surface

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1 **A multidisciplinary approach to the comparison of three contrasting**
2 **treatments on both lampenflora community and underlying rock surface**

3

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19

20

21 **Abstract**

22 Removing lampenflora, phototrophic organisms developing on rock surfaces in tourist cavities
23 due to the artificial lighting, is a challenge for sustainable and appropriate long-term management
24 of caves. Photosynthetic-based biofilms usually cause rock biodeterioration and an ecological
25 imbalance in cave ecosystems. In this work, a detailed investigation of the effects of the 3 most
26 commonly used lampenflora cleaning operations (NaClO, H₂O₂ and UVC) was carried out in
27 Pertosa-Auletta Cave (Italy). The application of NaClO showed good disinfection capability over
28 extended periods of time without causing any appreciable rock deterioration. The H₂O₂ treatment
29 showed to be corrosive for the rock surfaces covered with vermiculation deposits. The chemical
30 alteration of organic and inorganic compounds by H₂O₂ did not remove biomass, favoring
31 biofilm recovery after three months of treatment. Both NaClO and H₂O₂ treatments were
32 effective at removing photoautotrophs, although the bacterial phyla Proteobacteria and
33 Bacteroidetes as well as Apicomplexa and Cercozoa among the Eukaryotes, were found to be
34 resistant to these treatments. The UVC treatments did not show any noticeable effect on the
35 biofilms.

36
37 **Keywords:** Biofilms, Biodeterioration, Pertosa-Auletta Cave, Show caves, Biocides, Cave
38 management

40 **1 Introduction**

41 Artificial light in show caves causes the proliferation of lampenflora, green biofilms mainly
42 composed of photoautotrophic organisms (cyanobacteria, algae, ferns, mosses,...), causing
43 changes on speleothems and cave rock art, which are often the principal tourist attractions of such
44 environments (Mulec 2019). Except for the cave entrance area, photoautotroph organisms do not

45 grow in underground ecosystems where darkness prevails. Natural air streams and water flows, as
46 well as animals and humans, all contribute to the introduction of microbial cells, spores and
47 seeds, which proliferate easily in show caves, not only because of the artificial lighting systems
48 but also because of the shifts in relative humidity, temperatures and other favorable
49 environmental factors (Piano et al. 2015; Mulec 2019; Baquedano Estévez et al. 2019).

50 Besides the aesthetical changes of unnatural greenish coatings, lampenflora induces
51 biodeterioration of the rock and speleothem surfaces. Several of the lampenflora organisms
52 secrete organic acids that chemically dissolve the carbonate rock, or mechanically damage it,
53 through expanding anchor organs and like roots. Moreover, lampenflora causes an ecological
54 imbalance in the oligotrophic subterranean ecosystems by providing considerable organic supply
55 to other cave biota, replacing the autochthonous biodiversity by an invasive and opportunistic
56 community (Olson 2006; Mulec 2019; Baquedano Estévez et al. 2019).

57 To control and inhibit the “green disease” of show caves, besides acting on the lighting of the
58 cave itself (e.g., duration, intensity, positioning) (Piano et al. 2021), cave managers have applied
59 several physical and chemical cleaning methods to remove the phototrophic biomass from the
60 substrates and prevent further lampenflora development (Mulec and Kosi 2009; Baquedano
61 Estévez et al. 2019). Mechanical eradication using brush and water, liquid nitrogen application,
62 or led lighting systems with emission spectra distinct from lampenflora absorption spectra
63 (Muñoz-Fernández et al. 2021), or even the application of ecologically hazardous chemicals,
64 including herbicides, are the most often methods used against lampenflora proliferation (Mulec
65 2019; Baquedano Estévez et al. 2019). Among the most commonly cleaning method used today
66 in show caves is the application of commercial bleach (sodium hypochlorite, NaClO). This is a
67 low-cost and efficient method, but expensive in environmental terms by the release of chlorinated
68 organic compounds, potentially polluting the cave water cycle and biota (Meyer et al. 2017).

69 Only recently, the application of diluted hydrogen peroxide has been introduced as an eco-
70 friendlier remediation treatment for cave surfaces coated with green biofilms, because of the
71 absence of negative reaction by-products (Faimon et al. 2003; Mulec 2009; Trinh et al. 2018;
72 Baquedano Estévez et al. 2019). Another relatively popular treatment is a germicidal light system
73 with UVC irradiation, applied out of visiting hours. However, a perfect and definitive solution
74 has not yet been found, and the combination of different methods remains the most useful way to
75 control lampenflora (Grobbelaar 2000; Olson 2006; Mulec and Kosi 2009; Cigna 2012; Mulec
76 2019; Baquedano Estévez et al. 2019; Piano et al. 2021).

77 The actual efficacy and sustainability, in terms of lampenflora reduction and alterations caused
78 on the surfaces by such methods, are still not completely understood. This research provides an
79 extensive analysis on the effectiveness of the most widely employed methods for lampenflora
80 reduction and the associated substrate alterations, focusing on the taxonomic and functional
81 biodiversity of prokaryotic and eukaryotic communities. The effects of these treatments on the
82 composition of the organic fraction of the rock surface are still not well investigated (Baquedano
83 Estévez et al. 2019).

84 Here, a multidisciplinary approach is used to study the effect of different treatments against
85 lampenflora growing on bare rock and on vermiculated surfaces of a karstic limestone cave.
86 Vermiculations have been widely described in the literature as irregular sedimentary deposits
87 with peculiar morphologies developing on the walls, ceilings and floors of natural and artificial
88 underground environments (Addesso et al. 2019, 2020). The techniques used in this study
89 encompassed the application of photochemical efficiency measurements, microscopy, molecular
90 analysis (DNA) and, for the first time, direct analytical pyrolysis and thermogravimetric
91 techniques, which avoid any extraction or manipulation of the samples. A sector along the tourist
92 trail of the Pertosa-Auletta Cave (Campania, Italy) was studied with the aim of shedding light on

93 the potential damage induced on the substrates and to help in the design of better mitigation
94 strategies supporting decision-making of show cave managers.

95

96 **2 Methods**

97 ***2.1 Experimental plan and field activities***

98 To reduce the negative visible impact on the cave and the influence by visitors, the experimental
99 trial was set up in the final section of the tourist trail of the Pertosa-Auletta Cave (Campania
100 region, Italy) (Figure 1A). This cave section, temporarily closed to the public, has a DMX-
101 controlled RGBW led lighting system (OSRAM Licht AG; Munich, Germany), equipped with
102 motion detectors to comply with cave conservation programs and minimize the impact of visitors
103 to the cave environment. In this area, the motion detection sensors were turned off to ensure that
104 the surfaces under study would be illuminated for the whole eight hours of the cave's daily
105 opening. This system is ~ 3 km long, developed in pure Jurassic limestone, and has a mean
106 annual temperature of 16 °C. The tourist trail receives over 60.000 visitors per year, with a peak
107 season in August and a biological rest period in January (Addesso et al., 2019, 2020, 2022).
108 Eight representative wall areas (50 x 50 cm) covered by lampenflora were chosen, comprising
109 four bare surfaces (one area per treatment: control, NaClO, H₂O₂ and UV-C lamp) and four
110 surfaces with vermiculations (one area per treatment) (Figure 1B). Each 50 x 50 cm area was
111 split into 4 different sub-areas (25 x 25 cm) to create treatment replicates (Figure 1B,C,D) and
112 ensure representativeness of the treated section. Once a month, H₂O₂ 15% (Hydrogen peroxide
113 solution for analysis, Carlo Erba Reagents, Germany) and commercial bleach (NaClO),
114 commonly used during cleaning operations in show caves (certainly with other chemicals agents,
115 such as stabilizers, colorants), were applied on the studied surfaces. These chemical cleaning
116 treatments were applied using a laboratory wash bottle, spaying the solution homogeneously on

117 the colonized surfaces. In addition, a physical cleaning treatment was also tested, comprising
118 UVC irradiation for 8 hours during the night (technical and installation characteristics of the
119 UVC lamps are reported in Supplementary material, Table S1). Untreated rock surfaces for each
120 surface typology were also included as controls.

121 A photograph was taken for each sub-area within the whole area dimension ($n = 4$) using a digital
122 camera (SX620 Canon, Japan) before and after each treatment, for further image analyses. To
123 assess the biofilm's photosynthetic activity, *in situ* non-destructive photochemical efficiency of
124 maximal photosystem II (PSII) were carried out, before and after each treatment, using a portable
125 photosynthesis yield analyzer (MINI-PAM, WALTZ, Germany) equipped with a distance clip
126 holder (Distance Clip 2010A, WALTZ, Germany). One PSII measurement was conducted for
127 each sub-area within an entire area (50 x 50 cm) to obtain four measures per treatment ($n = 4$).
128 These measurements were carried out on 30 minutes dark-adapted surfaces, after covering the
129 surfaces with aluminium foil.

130 The experiment lasted a total of eight months (January to August 2020) with a break of three
131 months, from March to May 2020, due to the Covid19 pandemic. At the end of the experiment, a
132 representative sample was collected from each sub-area and for each surface typology and
133 treatment, scraping the surface with disposable and sterile scalpel blades and gathered it into
134 sterile Eppendorf tubes. Samples were stored in the laboratory at $-80\text{ }^{\circ}\text{C}$ until processing.

135

136 **2.2 Microscopy surveys**

137 Lampenflora chlorophyll fluorescence was visualized for samples collected at the end of the
138 assay, using a light and epifluorescence microscope (BX 61 OLYMPUS Corp., Japan) equipped
139 with a digital camera (DP73 Olympus Corp., Japan) and a specific DAPI filter.

140 Microphotographs were recorded at 10x magnification and image captures were processed using
141 Cell Sens software (OLYMPUS Corp. Shinjuku, Tokyo, Japan).
142 Oven-dried (50 °C) biofilm samples were sputter coated with gold and analyzed using a field
143 emission scanning electron microscope (FESEM) (FEI Teneo, Thermo Fisher, MA, USA), using
144 the secondary electron detection mode, with an acceleration voltage of 5 kV for ultra-high
145 resolution images.

146

147 ***2.3 Images and data analysis***

148 To macroscopically evaluate lampenflora evolution during treatments, the digital images were
149 processed using the ImageJ software obtaining a quantitative percentage value of the rock
150 surfaces covered with the photoautotrophic biofilms.

151 The differences in the single analyzed parameters based on the chlorophyll fluorescence and on
152 image analysis, were evaluated by three-way analyses of variance (three-way ANOVA), followed
153 by Tukey *post-hoc* tests, considering three fixed variables: the type of surface (bare or with
154 vermiculations), the time, and the type of treatments (no treatment in control areas, and
155 applications of NaClO, H₂O₂, and UVC irradiation).

156 All the statistical and graphical analyses were carried out in the R 4.0.0 programming
157 environment (R Core Team, 2020), with functions from the “vegan”, “agricolae”, “ggplot2”,
158 “dplyr”, “RColorBrewer” and “ggbreak” packages, and using the open-source vector graphics
159 editor Inkscape 0.92.

160

161 ***2.4 Molecular analysis***

162 The DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hiden, Germany) was used to extract total
163 DNA according to the producer’s protocol. The DNA amount was determined using a Qubit 4.0

164 Fluorometer (Invitrogen, Waltham, MA, USA). The extracted DNA (with a minimum
165 concentration of ~ 0.1 ng/ μ L) was analyzed via next-generation sequencing (NGS) targeting the
166 V3–V4 hypervariable region of bacterial 16S rRNA gene and V4 of Eukaryotes 18S rRNA gene,
167 using Illumina MiSeq 2 \times 300 paired-end, according to Macrogen (Seoul, Korea) library
168 preparation protocol. The raw data were quality checked, trimmed and clustered in operational
169 taxonomic units (OTUs) with a 97% similarity threshold using QIIME2 microbiome
170 bioinformatics platform (Bolyen et al. 2019) with DADA2 sample inference program (Callahan
171 et al. 2016). Taxonomic identification was carried out using SILVA v.132. and NCBI databases
172 for Bacteria and Eukaryotes, respectively.

173

174 ***2.5 Analytical pyrolysis***

175 Changes in the molecular chemical structure of rock surfaces were studied by pyrolysis-gas
176 chromatography/mass spectrometry (Py-GC–MS). A double-shot microfurnace pyrolyser (model
177 2020i, Frontier Laboratories, Fukushima, Japan) attached to a GC-MS Agilent 6890N (Agilent
178 Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973 mass selective detector system,
179 was used for the direct analysis of samples. Finely ground samples (c. 10 mg) were placed in
180 small crucible capsules and introduced into a preheated micro-furnace at 400 °C for 1 min. The
181 pyrolysis products were directly injected into the gas chromatograph inlet line heated at 250 °C to
182 prevent condensation. The GC was equipped with a HP-5ms-UI, low polar-fused silica (5%-
183 Phenyl-methylpolysiloxane) (J&W Scientific, Folsom, CA, USA) capillary column of 30 m \times
184 250 μ m \times 0.25 μ m film thickness (Ref. DB-5). Chromatographic conditions were similar to those
185 described in Miller et al. (2022). Compound assignment was achieved by considering diagnostic
186 ions for the main homologous series, via low-resolution MS and via comparison with published
187 and stored data in NIST and Wiley libraries. A semi-quantification of the products released by

188 analytical pyrolysis was done for each sample by converting the peak areas to a percentage of the
189 total chromatographic area. Minor compounds with 0.2% of the total chromatographic area were
190 excluded.

191

192 **2.6 Thermal analysis**

193 Thermogravimetry (TG), derivative TG, and differential scanning calorimetry (DSC) of dried (40
194 °C) samples were conducted using Discovery series SDT 650 simultaneous DSC/TGA instrument
195 (TA Instruments Inc., New Castle, DE, USA) under a N₂ flow rate of 50 ml min⁻¹. Thus, 5 mg of
196 each sample were placed in Alumina cups without cover and heated from 50 to 650 °C at a
197 heating rate of 20 °C min⁻¹. TG, dTG curves, mass loss and calorimetry data were obtained via
198 TRIOS software (TA Instruments, New Castle, DE, USA). Experiments were performed twice
199 with a reproducibility error ≤0.5 %. The weight loss of the decomposed materials was divided
200 into four groups in terms of the proportions of: W1 (moisture and very labile Organic Matter
201 (OM)), W2 (labile OM), W3 (intermediate OM), W4 (recalcitrant OM) components.

202

203

204 **3. Results**

205 **3.1 Image analyses and maximal PSII photochemical efficiency**

206 The image analysis approach allowed monitoring the development of lampenflora on the four
207 treated surfaces (Figure 2). Differences on the adhesion of the greenish biofilms on the bare and
208 vermiculated surfaces were observed, which were related to the nature of the rock surfaces. On
209 the limestone rock substrate (bare surfaces), thin, homogeneously distributed, and well-adhered
210 biofilms were observed. In contrast, green biofilms were heterogeneously distributed on the
211 vermiculation deposits and were easily removed from the surface. Both types of surfaces, bare

212 (Figure 2A) and with vermiculations (Figure 2B) showed a complete disappearance of green
213 biofilms after NaClO treatment, whereas the application of H₂O₂ solely caused a slight decrease
214 of the green biomass. Control and UVC-irradiated surfaces exhibited the same trend, indicating
215 no biocidal action due to UVC radiation exposure. Moreover, the whole area covered by
216 lampenflora with vermiculations showed a gradual decline, irrespective of the treatment applied,
217 even for the control samples.

218 The maximal PSII photochemical efficiency, measured before and after the treatments on the two
219 surface typologies covered by lampenflora (bare (Figure 2C) and with vermiculations (Figure
220 2D), is also shown in Figure 2. At time 0, Fv/Fm mean values for the bare surface and that
221 covered with vermiculations were 0.695 and 0.744, respectively. After the first chemical
222 treatment, either with H₂O₂ or NaClO, Fv/Fm mean values dropped close to 0, remaining at this
223 very low value up to a week after the second treatment, indicating a nearly complete reduction of
224 biological activity. However, following the three months break due to the Covid19 lockdown,
225 there was a slight recovery of lampenflora on H₂O₂-treated surfaces, but Fv/Fm mean values
226 dropped again after the third treatment. No detectable effect occurred in relation to the biofilm
227 photosynthetic activity on the surfaces exposed to UVC radiation, exhibiting a trend similar to
228 the control areas. The output parameters obtained by the three-way ANOVAs are reported in
229 Table 1. In both cases, the amount of lampenflora (Table 1A) and the Fv/Fm values (Table 1B)
230 differed significantly in time and with the typology of treatments ($P < 0.001$).

231

232 **3.2 Microscopy observations**

233 Light microscopy, epifluorescence and FESEM examinations of bare surfaces covered by
234 lampenflora and treated with the different methods are reported in Figures 3 and 4. The untreated
235 photosynthetic-based community was mainly composed of filamentous photoautotrophic

236 microorganisms (Figure 3A-C), as revealed by the *in vivo* pigment fluorescence (red filaments in
237 Figure 3B). Visual changes on community structure or chlorophyll autofluorescence were not
238 observed for the UVC-treated surfaces (Figure 3D-F), which again showed a trend similar to the
239 control samples. The surface treated with NaClO appeared to be devoid of any biomass, that was
240 eliminated by this chemical solution without causing significant damage to the substrate (Figure
241 3G-I). The H₂O₂ solution caused the death of the biological community, but not its removal from
242 the substrate, as residual organic matter was observed on the treated surface (Figure 3J-L). The
243 same behavior occurred for the treated lampenflora on surfaces covered with vermiculations
244 (Figure 4). However, the photosynthetic-based community on this surface was almost absent as
245 evidenced by the low biomass and chlorophyll autofluorescence intensity in the control samples
246 (Figure 4A-C). This was also observed for the UVC- (Figure 4D-F) and NaClO-treated surfaces
247 (Figure 4G-I). The H₂O₂-treated surfaces showed evidence of corrosion caused by this treatment
248 (Figure 4J-L), as revealed by the presence of etch pits on the mineral substrate (Figure 4L).

249

250 **3.3 Lampenflora community composition**

251 A total of 1428 OTUs were obtained for the 8 samples for Bacteria, and 467 OTUs for
252 Eukaryotes. The major phylum in the bacterial community of the bare control surface (Figure 5A)
253 was the Cyanobacteria (41.2%) dominated by the class Cyanophyceae (41.2%) (Figure 5B) and
254 the order Nostocales (39.3%) (Figure 5C). This was followed by the phylum Proteobacteria
255 (36.0%), dominated by Alpha- (15.1%), Beta- (8.8%) and Gamma-proteobacteria (8.6%) classes
256 (Figure 5B). Members belonging to the phyla Acidobacteria (5.0%), Bacteroidetes (3.3%),
257 Actinobacteria (2.4%), Firmicutes (2.1%), Nitrospirae (1.5%) and to unclassified phyla (6.0%)
258 were also detected (Figure 5A). The control samples from the surface with vermiculations
259 (Figure 5A) exhibited a bacterial composition similar to the bare surface, with phylum

260 Proteobacteria (59.8%), dominated by the classes Gamma- (24.7%), Beta- (24.7%) and Alpha-
261 proteobacteria (7.1%) (Figure 5B), followed by unclassified Bacteria (9.9%) and several phyla:
262 Firmicutes (9.0%), Nitrospirae (5.4%), Bacteroidetes (4.8%), Acidobacteria (3.3%),
263 Actinobacteria (2.8%), Chloroflexi (1.8%), and Gemmatimonadetes (1.2%). Members of the
264 phylum Cyanobacteria were detected with a relative abundance of 0.4%.

265 The most abundant phyla of the lampenflora from the bare surfaces treated with UVC were
266 (Figure 5A): Proteobacteria (33.7%), Cyanobacteria (32.0%), Bacteroidetes (11.6%), Firmicutes
267 (6.5%), unclassified Bacteria (5.2%), Acidobacteria (3.0%), Chloroflexi (2.4%), Synergistetes
268 (2.0%) and Actinobacteria (1.1%). The phylum Proteobacteria was dominated by classes Alpha-
269 (14.4%), Delta- (7.7%), Beta- (6.4%) and Gamma-proteobacteria (5.5%), while the phylum
270 Cyanobacteria was dominated by the class Cyanophyceae (32.0%) (Figure 5B) and by the order
271 Nostocales (31.3%) (Figure 5C). Samples obtained from the surface with vermiculations treated
272 with UVC, at the phylum level (Figure 5A), were almost totally composed of Proteobacteria
273 (74.4%), represented by classes Beta-(38.9%), Gamma- (22.8%), Alpha- (9.56%) and Delta-
274 proteobacteria (3.1%) (Figure 5B), followed by phyla Acidobacteria (6.1%), unclassified Bacteria
275 (5.7%), Chloroflexi (3.3%), Nitrospirae (2.7%), Gemmatimonadetes (2.5%), Firmicutes (1.8%),
276 Actinobacteria (1.6%), and Bacteroidetes (1.0%).

277 Samples collected from the bare surfaces treated with NaClO and H₂O₂ exhibited similar
278 microbial communities mainly composed of Bacteroidetes (56.9% and 50.3%, respectively)
279 (Figure 5A), and represented by Flavobacteriia (55.9 and 44.2%, respectively) class (Figure 5B)
280 and, at the order level, by Flavobacteriales (55.9 and 44.2%, respectively) (Figure 5C). For both
281 treatments, the relative abundance of Proteobacteria was 40.8 and 41.8%, respectively (Figure
282 5A), mainly dominated by Gamma- (32.0 and 23.8%, respectively), Alpha- (0.2 and 11.2%,
283 respectively) and Beta-proteobacteria (8.3 and 5.4%, respectively) classes (Figure 5B).

284 Interestingly, the entire bacterial community of the sample from the vermiculated surfaces treated
285 with NaClO was composed of Proteobacteria (100%) phylum (Figure 5A), primarily represented
286 by the Gamma-proteobacteria (99.4%) class (Figure 5B) and by Pseudomonadales (99.4%), at
287 order level (Figure 5C). The vermiculated surfaces treated with H₂O₂ were more biodiverse
288 (Figure 5A), composed of Proteobacteria (46.5%), represented by Alpha- (27.3%), Beta- (8.7%),
289 Gamma- (7.7%), and Delta-proteobacteria (1.8%) classes (Figure 5B), and by Bacteroidetes
290 (34.2%) phylum, dominated by Flavobacteriia (18.1%) and Sphingobacteriia (6.9%) classes,
291 followed by the phyla Firmicutes (5.1%), Verrucomicrobia (3.8%), Actinobacteria (2.5%), and
292 Gemmatimonadetes (1.1%).

293 Regarding the Eukaryotic community (Figure 5D-F), the controls, as well as the UVC irradiated
294 samples, from bare and vermiculated surfaces, were mostly represented by the phylum
295 Streptophyta (86.3 and 99.0% - 92.8 and 99.3%, respectively for control and UVC treatments)
296 (Figure 5D). This was dominated by Bryopsida class (86.2 and 98.5% - 92.7 and 99.2%,
297 respectively for control and UVC treatments) (Figure 5E) and, at order level, by Pottiales (86.2
298 and 98.5% - 92.7 and 99.2%, respectively for control and UVC) (Figure 5F). The bare surfaces
299 and those with vermiculations treated with NaClO were mainly characterized by Streptophyta
300 (91.4 and 95.8%, respectively) phylum (Figure 5D), dominated by the classes Magnoliopsida
301 (76.2 and 94.2%, respectively) and Bryopsida (15.1 and 1.3%, respectively) (Figure 5E), and the
302 orders by Lamiales (68.1 and 4.2%, respectively), Pottiales (15.1 and 1.3%, respectively), and
303 Poales (7.6 and 74.3%, respectively) (Figure 4F). The major phyla composing the eukaryotic
304 community of the bare and vermiculated surfaces treated with H₂O₂ were represented by
305 Cercozoa (59.3 and 4.3%, respectively), unclassified Eucaryota (19.0 and 54.6%, respectively),
306 unclassified DNA sequences (13.9 and 5.7%, respectively), Apicomplexa (5.6 and 18.1%,
307 respectively), and Streptophyta (0.6 and 17.1%, respectively) (Figure 5D).

308

309 **3.4 Molecular composition of lampenflora**

310 A total of 59 different organic compounds were detected from the pyro-chromatograms of the
311 samples and their relative abundances were calculated from the peak areas. The compounds were
312 categorized into 5 main product groups with a similar nature or known origin: Alkyl compounds
313 (ALK); aromatic compounds (ARO), polysaccharide-derived (PS), nitrogen compounds (N), and
314 contaminants (CONT) (Figure 6, Table S2). All the pyro-chromatograms from the vermiculated
315 surfaces were dominated by polysaccharide-derived substances and alkyl structures. Furthermore,
316 no changes in chemical composition were observed after both H₂O₂ and NaClO treatments. In
317 contrast, clear differences were found for the bare surfaces. Samples treated with H₂O₂ showed
318 the presence of a series of *n*-alkane/alkene doublets, which were not present in the control
319 samples. On the contrary, the application of NaClO resulted in the practically total absence of
320 organic molecules, except for the persistence of a few ALK molecules and several small peaks
321 corresponding to ARO compounds. It is remarkable that the NaClO treatment caused the total
322 elimination of PS, which were dominant compounds in all other treatments.

323

324 **3.5 Thermal analysis**

325 The total and relative weight loss of the biofilms from both bare and vermiculated surfaces for
326 the different treatments are shown in Table 2. All treated samples (bare and with vermiculations)
327 were characterized by a lower weight loss (2-to-4 times lower) than the control samples. The
328 latter displays a high abundance of very labile organic matter (W1). The differential scanning
329 calorimetry (DSC) data revealed an increase of the thermal resistance of the treated bare biofilms,
330 which is evidenced by the shift of the main exothermic peak to higher temperatures when
331 compared with the control samples. Biofilms with vermiculations showed a greater relative

332 abundance of the most thermally labile fraction (W1) than the corresponding bare sample, which
333 agreed with the presence of labile organic remains from cyanobacteria and algae depositions and
334 a decrease of the released heat per unit of organic matter (Q' released).

335

336 **4. Discussion**

337 Solving the problem of lampenflora in show caves is a challenge and a priority for the
338 preservation and sustainable management of these fragile ecosystems. Fine-tuning surface
339 cleaning methods to remove this “green disease”, prevent its growth without compromising the
340 wall integrity and the underground habitat, is desired by most show cave managers (Chiarini et
341 al. 2022). However, little is known about the effects of the most used lampenflora removal
342 methods (ie, physical cleaning, UVC irradiation, and chemical treatments with NaClO, H₂O₂ or
343 other products), both on lampenflora metabolism and on the treated rock surface composition.
344 Furthermore, there is no standardized action to quantitatively and qualitatively monitor treatment
345 efficacy over time (Baquedano Estévez et al. 2019). The results of this study offer a useful
346 comparison of the processes activated on surfaces by three commonly employed chemical-
347 physical methods of lampenflora removal (UVC radiation, NaClO and H₂O₂), shedding light on
348 microbial community evolution following the treatments and potential damage on the substrates.
349 The maximal PSII photochemical efficiency measures (quantum yield) proved to be a valid *in*
350 *situ* and non-destructive method to monitor the metabolic dynamics of green lampenflora in
351 caves, representing a proxy of the physiological status of the community related to photosynthetic
352 activity (Figuroa et al. 2017). The Fv/Fm values recorded on the non-treated green biofilms
353 from the Pertosa-Auletta Cave were in line with those of the lampenflora growing in other caves,
354 such as Cango Cave (South Africa) (mean value 0.74; Grobbelaar 2000) and in La Glacière Cave
355 (France) (mean value 0.70; Pfendler et al. 2017). The chemical treatments (NaClO and H₂O₂)

356 annealed completely the quantum yields already after the first application, due to the oxidation
357 reactions of the organic substrates (Faimon et al. 2003). As revealed by microscopy images,
358 lampenflora was totally removed after NaClO treatment, whereas it was severely damaged when
359 using H₂O₂. In fact, after three months without treatments, the areas treated with H₂O₂ showed
360 signs of lampenflora recovery, whereas those that were cleaned with NaClO remained clean. The
361 visible re-colonization was different for the two chemical treatments: NaClO acted without
362 damaging neither bare nor vermiculated surfaces, whereas when H₂O₂ was used, a slight
363 brightening was observed, but also all the death organic matter remained on the surfaces. This
364 latter effect may represent an energy source for the cave biota, as previously described by Mulec
365 (2019). Moreover, H₂O₂ treatment was visibly corrosive for vermiculations, mainly composed of
366 calcite (Addesso et al. 2019), producing an effervescent reaction, indicative of oxidation of
367 organic matter and carbonates, producing CO₂ that in turn lead to dissolution phenomena (Trinh
368 et al. 2018). This was already observed by Faimon et al. 2003. Therefore, if on one hand, a higher
369 concentration might be needed to eliminate the organic matter, on the other, this leads to an
370 increase of its corrosive action, requiring a chemical re-equilibration of the solution (which needs
371 to be put in contact with CaCO₃ powder before its application). Mulec (2014) proposed using a
372 carbonate/bicarbonate buffer in the final peroxide solution to remove lampenflora because it
373 preserves pH levels not corrosive for cave formations. As previously suggested by Trinh et al.
374 (2018), after a H₂O₂ treatment, a waterjet or brushing would be required for the complete
375 removal of the biomass from the surfaces. Such mechanical treatment would only be feasible on
376 hard surfaces like granite (Pozo-Antonio and Sanmartín 2018), and not on soft surfaces, such as
377 those containing vermiculations or pasty moonmilk.

378 The scarce lampenflora colonization of surfaces with vermiculations, which was confirmed by
379 microscopy observations, can be explained by the switching off of one of the three lamps

380 installed in this section of the cave during the period of observation, without the possibility to
381 restore it again. This confirms that direct light represents the primary driver determining green
382 biofilm growth (Sanmartín et al. 2012; Piano et al. 2015, 2021).

383 In relation to the taxonomic community composition, the most abundant photosynthetic-based
384 taxon in the Pertosa-Auletta Cave lampenflora biofilms in the untreated bare surface, was
385 Cyanobacteria, specifically by the aerophytic filamentous cyanobacterial species *Brasilonema*
386 *angustatum* (39.3%), belonging to the Scytonemataceae family, isolated from the island of Oahu,
387 Hawaii (Vaccarino and Johansen, 2012), and by *Aerosakkonema funiforme* (1.8%), gas-
388 vacuolated oscillatoriid cyanobacterium, isolated from freshwater (Thu et al. 2012). It is well
389 known that Cyanobacteria are among the pioneering organisms involved in lampenflora
390 development (Popović et al. 2017; Mulec 2019; Baquedano Estévez et al. 2019; Havlena et al.
391 2021). The vermiculated untreated surface revealed very low amounts of photoautotrophic
392 organisms (< 1%), being mainly composed of several phyla commonly found in cave
393 environments, such as Proteobacteria, Nitrospira, Firmicutes, Actinobacteria, and Acidobacteria
394 (Tomczyk-Żak and Zielenkiewicz 2016; Adesso et al. 2020). After the application of both
395 chemical treatments on the bare surfaces, the photoautotrophs were eliminated, but not the
396 bacterial population, mainly Proteobacteria and Bacteroidetes, also present on vermiculated
397 surfaces treated with H₂O₂, whereas those treated with the commercial bleach (NaClO) only
398 showed Proteobacteria. The inefficacy of the chemical treatments in removing non-
399 photosynthetic microorganisms can be related to the presence of microbial species that can
400 tolerate NaClO. In fact, Proteobacteria followed by Bacteroidetes have been identified as the
401 main cause of membrane fouling in membrane bioreactors systems after NaClO backwashing
402 (Wang et al. 2014, 2019). The eukaryotic community from both the untreated surface types was
403 almost exclusively composed of *Ephemerum spinulosum*, which are plants belonging to the

404 Pottiaceae family, typically found in very damp environments (Ignatov et al. 2013). The H₂O₂
405 treatment was more effective on eliminating members of the phylum Streptophyta than NaClO
406 (having no effect), with a residual presence of Apicomplexa, a group of unicellular protists, and
407 Cercozoa phylum.

408 Despite several studies reporting its efficiency (Borderie et al. 2015; Pfendler et al. 2017), the
409 treatment with UVC lamps, once a month, did not produce alterations, neither in the community
410 composition nor in the substrate structure. This could be likely linked to a too mild and
411 ineffective treatment. An increase of time exposition of surfaces from one to four times a month
412 probably would have determined appreciable effects, but unfortunately, for logistic reasons
413 related also to the Covid19 pandemic, further trials of this method were not possible.

414 Analytical pyrolysis was useful to detect shifts in the molecular composition caused by the
415 different chemical treatments on the surfaces, highlighting different macromolecular assemblages
416 in the samples. The pyrolysates of untreated bare surfaces were found mainly composed of non-
417 specific ARO compounds, commonly observed in natural biomolecules (Miller et al. 2016), PS
418 derived compounds, mainly furan compounds and their derivatives, and minor proportions of
419 ALK, mainly C₁₁ to C₂₁ branched structures with a probable biogenic origin in archaea or algae
420 (Fowler and Douglas 1987; Shiea et al. 1990) and N compounds (mainly pyridine), with a
421 probable polypeptide or protein origin (Saiz-Jimenez et al. 2021).

422 The analysis of the chemically treated surfaces provided interesting information mainly about the
423 effects of NaClO on bare surfaces with a sharp reduction in the number of released compounds
424 and a complete absence of the biogenic PS and N compounds, pointing to a high effectivity of
425 this treatment. The effect of H₂O₂ treatment, produced a more diverse chromatogram with more
426 compounds and in higher abundance, mainly PS derived, N compounds and normal and branched
427 alkanes with a probable microbial origin. No noticeable differences were observed in the

428 pyrolyzates of control and treated vermiculated surfaces. They are dominated by polysaccharide-
429 derived compounds and *n*-alkanes, which are the major organic compounds of cyanobacteria,
430 algae, non-vascular plants and moss.

431 Concerning thermal analyses, the sharp reduction of the total weight loss of the treated biofilms
432 and the transfer of the relative weight loss from W1 (labile OM) to W3 and W4 for the treated
433 biofilms compared with the control samples pointed out the partial elimination of lampenflora
434 organic remains by the treatments. The thermal degradation of control samples is characterized
435 by the high relative abundance of very labile OM, typically composed of polysaccharides (the
436 main constituent of extracellular polymeric substances), whereas the thermograms of treated
437 biofilms showed a great relative abundance of thermally recalcitrant OM, typically composed of
438 aromatic structures. In contrast, the TG curves of NaClO-treated biofilms were characterized by
439 the lowest relative abundance of very labile and labile OM as well as the greatest presence of
440 recalcitrant OM remains, suggesting a more effective removal of fresh biofilms. The dTg and
441 DSC for the control at bare surface showed peaks with maxima at 356 °C, whereas the treated
442 biofilms showed maxima ranging from 404 to 425 °C. Both peaks fit perfectly with those
443 reported for the combustion of microalgae and fungi, and are attributable to combustion of
444 protein and lipids, respectively (Kang and Yoon 2015).

445

446 **5. Conclusions**

447 The results provided relevant and useful information concerning the efficacy of the most
448 employed physical (UVC) and chemical (NaClO, H₂O₂) control and removal methods of
449 lampenflora in show caves. This work offers a comprehensive assessment of biofilm physiology,
450 chemical composition, as well as of the potential deterioration processes of the underlying rock
451 substrates in response to their applications. Commercial bleach (NaClO) treatment seemed to be

452 the most efficient method in relation to both surface sterilization and visible cleaning over long
453 time, with unaltered underlying rock substrates. However, the toxicity of chlorine compounds is
454 known and an important drawback, requiring the use of diluted solutions, thus limiting the
455 efficacy of this method. The H₂O₂ treated surfaces showed a recovery of lampenflora after three
456 months without applications, and evident rock dissolution processes activated on surfaces.
457 Indeed, H₂O₂ treatment promoted the release of alkyl chemical structures and a visible
458 deterioration of vermiculation deposits. In addition, the organic matter was not eliminated by the
459 application of H₂O₂, and consequently the remaining organic matrix would need to be removed
460 through brushing or water jets in order to avoid undesirable effects on the ecological balance of
461 the caves. Based on the conditions tested in this work, the UVC irradiation treatment was not an
462 effective method for the removal of lampenflora.

463

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480

481 **Declaration of interest statement**

482 The authors report there are no competing interests to declare.

483

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591 **TABLES**

592 **Table 1.** Output parameters of the three-way ANOVAs using amount percentage values of
 593 biofilms on surfaces (a) and Fv/Fm data (b), considering the three fixed variables, the type of
 594 surface, the time and the treatments.

595 **a.**

	Df	F value	Pr(>F)
Surface type	1	3.214	0.0771
Time	1	46.056	2.45e-09
Treatment	3	64.747	< 2e-16
Residuals	74		

601

602 **b.**

603

	Df	F value	Pr(>F)
Surface type	1	0.826	0.3675
Time	9	8.196	4.63e-08
Treatment	3	168.252	< 2e-16
Residuals	66		

609

610 **Table 2.** Comparative thermogravimetry (TG) of the biofilm samples from bare and vermiculated
611 surfaces for the control and the different treatments. Total weight loss for the temperature interval
612 50–650 °C ($\% \pm 1\%$), weight losses and relative weight losses for the temperature intervals 50–
613 120 °C, 120–200 °C, 200–400 °C, and 400–600 °C, and temperature of the main exothermic
614 peaks.

615

<i>TG</i>		Bare surface				With vermiculations			
		Control	UVC	NaClO	H ₂ O ₂	Control	UVC	NaClO	H ₂ O ₂
Moisture and very labile OM-W1	50–120 °C	2.1	0.4	0.3	0.3	2.8	0.9	0.4	0.4
Labile OM-W2	120–200 °C	0.7	0.2	0.2	0.1	0.9	0.4	0.1	0.2
Int OM-W3	200–400 °C	2.1	0.8	0.7	0.6	2.2	1.3	0.5	0.7
Recalcitrant OM-W4	400–600 °C	3.1	1.0	1.1	0.7	3.6	1.4	1.5	0.8
Total weight loss	50–650 °C	8.0	2.4	2.3	1.7	9.5	3.9	2.5	2.1
<i>Relative Weight Loss (%)</i>									
Moisture and very labile OM-W1	50–120 °C	26	16	13	18	29	22	16	21
Labile OM-W2	120–200 °C	9	8	8	6	9	9	4	9
Int OM-W3	200–400 °C	27	35	31	34	24	33	20	32
Recalcitrant OM-W4	400–600 °C	39	41	48	42	38	36	60	38

616

617

618 **FIGURE CAPTIONS**

619 **Figure 1.** A. Pertosa-Auletta Cave map, showing in green the tourist trail. Red circle indicates
620 the chosen section for treatments. B. Schematic experimental plan. C. Delimited area of a bare
621 surface covered by lampenflora. D. Delimited area of a surface with vermiculations covered by
622 lampenflora.

623
624 **Figure 2.** Maximal PSII photochemical efficiency (F_v/F_m) and percentage of surface covered
625 area by lampenflora, measured before and after the treatments (control, orange; H_2O_2 , green;
626 $NaClO$, light blue; UVC, violet) on bare rock surfaces (respectively, A and C) and those covered
627 with vermiculations (respectively, B and D). Different letters indicate significant (for $\alpha=0.05$)
628 differences among treatments over time (small letters) and treatment typologies (capital letters),
629 according to the Tukey post-hoc tests.

630
631 **Figure 3.** Representative microscopy images of the biofilms on the bare surfaces: without
632 treatment, control (A-C); treated with UVC irradiation (D-F); treated with $NaClO$ (G-I), and
633 treated with H_2O_2 (J-L). The corrosion features as well as the collapsed biological masses due to
634 the H_2O_2 treatments are indicated with arrows (L).

635
636 **Figure 4.** Representative microscopy images of the biofilms on the surfaces with vermiculations:
637 without treatment, control (A-C); treated with UVC irradiation (D-F); treated with $NaClO$ (G-I),
638 and treated with H_2O_2 (J-L). The corrosion features as well as the collapsed biological masses
639 due to the H_2O_2 treatments are indicated with arrows (L).

640

641 **Figure 5.** Bacteria and Eukaryotes composition of the lampenflora from bare (A-C) and
642 vermiculated (D-F) surfaces for each treatment type (control, UVC, NaClO, H₂O₂); the barplots
643 show the relative abundances (%) at phylum (A,D), class (B,E), and order (C,F) levels.

644

645 **Figure 6.** A. Representative annotated pyrograms (Py-GC/MS). B. Relative abundance (%) by
646 compound group in the biofilms from bare and vermiculated surfaces for control, H₂O₂ and
647 NaClO treatments. The numbers on the peaks correspond to those listed in Table S2.