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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
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21 Abstract

22 Removing lampenflora, phototrophic organisms developing on rock surfaces in tourist cavities due to the artificial lighting, is a challenge for sustainable and appropriate long-term management 23 of caves. Photosynthetic-based biofilms usually cause rock biodeterioration and an ecological 24 imbalance in cave ecosystems. In this work, a detailed investigation of the effects of the 3 most 25 commonly used lampenflora cleaning operations (NaClO, H_2O_2 and UVC) was carried out in 26 27 Pertosa-Auletta Cave (Italy). The application of NaClO showed good disinfection capability over extended periods of time without causing any appreciable rock deterioration. The H_2O_2 treatment 28 29 showed to be corrosive for the rock surfaces covered with verniculation deposits. The chemical 30 alteration of organic and inorganic compounds by H₂O₂ did not remove biomass, favoring biofilm recovery after three months of treatment. Both NaClO and H₂O₂ treatments were 31 32 effective at removing photoautotrophs, although the bacterial phyla Proteobacteria and Bacteroidetes as well as Apicomplexa and Cercozoa among the Eukaryotes, were found to be 33 34 resistant to these treatments. The UVC treatments did not show any noticeable effect on the biofilms. 35

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Keywords: Biofilms, Biodeterioration, Pertosa-Auletta Cave, Show caves, Biocides, Cave
management

39

40 **1 Introduction**

Artificial light in show caves causes the proliferation of lampenflora, green biofilms mainly
composed of photoautotrophic organisms (cyanobacteria, algae, ferns, mosses,...), causing
changes on speleothems and cave rock art, which are often the principal tourist attractions of such
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).

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

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

Here, a multidisciplinary approach is used to study the effect of different treatments against 84 lampenflora growing on bare rock and on vermiculated surfaces of a karstic limestone cave. 85 86 Vermiculations have been widely described in the literature as irregular sedimentary deposits with peculiar morphologies developing on the walls, ceilings and floors of natural and artificial 87 underground environments (Addesso et al. 2019, 2020). The techniques used in this study 88 encompassed the application of photochemical efficiency measurements, microscopy, molecular 89 analysis (DNA) and, for the first time, direct analytical pyrolysis and thermogravimetric 90 techniques, which avoid any extraction or manipulation of the samples. A sector along the tourist 91 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 mitigation94 strategies supporting decision-making of show cave managers.

95

96 **2 Methods**

97 2.1 Experimental plan and field activities

To reduce the negative visible impact on the cave and the influence by visitors, the experimental 98 99 trial was set up in the final section of the tourist trail of the Pertosa-Auletta Cave (Campania region, Italy) (Figure 1A). This cave section, temporarily closed to the public, has a DMX-100 controlled RGBW led lighting system (OSRAM Licht AG; Munich, Germany), equipped with 101 102 motion detectors to comply with cave conservation programs and minimize the impact of visitors to the cave environment. In this area, the motion detection sensors were turned off to ensure that 103 104 the surfaces under study would be illuminated for the whole eight hours of the cave's daily opening. This system is ~ 3 km long, developed in pure Jurassic limestone, and has a mean 105 annual temperature of 16 °C. The tourist trail receives over 60.000 visitors per year, with a peak 106 season in August and a biological rest period in January (Addesso et al., 2019, 2020, 2022). 107 Eight representative wall areas (50 x 50 cm) covered by lampenflora were chosen, comprising 108 four bare surfaces (one area per treatment: control, NaClO, H_2O_2 and UV-C lamp) and four 109 110 surfaces with vermiculations (one area per treatment) (Figure 1B). Each 50 x 50 cm area was split into 4 different sub-areas (25 x 25 cm) to create treatment replicates (Figure 1B,C,D) and 111 ensure representativeness of the treated section. Once a month, H₂O₂ 15% (Hydrogen peroxide 112 113 solution for analysis, Carlo Erba Reagents, Germany) and commercial bleach (NaClO), commonly used during cleaning operations in show caves (certainly with other chemicals agents, 114 such as stabilizers, colorants), were applied on the studied surfaces. These chemical cleaning 115 treatments were applied using a laboratory wash bottle, spaying the solution homogeneously on 116

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

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

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

135

136 **2.2** *Microscopy surveys*

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

Microphotographs were recorded at 10x magnification and image captures were processed using
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

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

image analysis, were evaluated by three-way analyses of variance (three-way ANOVA), followed

by Tukey *post-hoc* tests, considering three fixed variables: the type of surface (bare or with

vermiculations), the time, and the type of treatments (no treatment in control areas, and

applications of NaClO, H_2O_2 , and UVC irradiation).

156 All the statistical and graphical analyses were carried out in the R 4.0.0 programming

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

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 $\sim 0.1~ng/\mu L)$ 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 2×300 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.

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174 2.5 Analytical pyrolysis

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

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

191

192 **2.6** *Thermal analysis*

Thermogravimetry (TG), derivative TG, and differential scanning calorimetry (DSC) of dried (40 193 194 °C) samples were conducted using Discovery series SDT 650 simultaneous DSC/TGA instrument (TA Instruments Inc., New Castle, DE, USA) under a N₂ flow rate of 50 ml min⁻¹. Thus, 5 mg of 195 each sample were placed in Alumina cups without cover and heated from 50 to 650 °C at a 196 heating rate of 20 °C min⁻¹. TG, dTG curves, mass loss and calorimetry data were obtained via 197 TRIOS software (TA Instruments, New Castle, DE, USA). Experiments were performed twice 198 199 with a reproducibility error ≤ 0.5 %. The weight loss of the decomposed materials was divided into four groups in terms of the proportions of: W1 (moisture and very labile Organic Matter 200 (OM)), W2 (labile OM), W3 (intermediate OM), W4 (recalcitrant OM) components. 201 202 203

3. Results

205 3.1 Image analyses and maximal PSII photochemical efficiency

The image analysis approach allowed monitoring the development of lampenflora on the four treated surfaces (Figure 2). Differences on the adhesion of the greenish biofilms on the bare and vermiculated surfaces were observed, which were related to the nature of the rock surfaces. On the limestone rock substrate (bare surfaces), thin, homogeneously distributed, and well-adhered biofilms were observed. In contrast, green biofilms were heterogeneously distributed on the vermiculation deposits and were easily removed from the surface. Both types of surfaces, bare (Figure 2A) and with vermiculations (Figure 2B) showed a complete disappearance of green
biofilms after NaClO treatment, whereas the application of H₂O₂ solely caused a slight decrease
of the green biomass. Control and UVC-irradiated surfaces exhibited the same trend, indicating
no biocidal action due to UVC radiation exposure. Moreover, the whole area covered by
lampenflora with vermiculations showed a gradual decline, irrespective of the treatment applied,
even for the control samples.

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

231

232 **3.2** *Microscopy observations*

Light microscopy, epifluorescence and FESEM examinations of bare surfaces covered by

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

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

249

250 **3.3** Lampenflora community composition

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

- 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:
- Firmicutes (9.0%), Nitrospirae (5.4%), Bacteroidetes (4.8%), Acidobacteria (3.3%),
- 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
- 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-
- 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)
- (Figure 5A), and represented by Flavobacteriia (55.9 and 44.2%, respectively) class (Figure 5B)
- and, at the order level, by Flavobacteriales (55.9 and 44.2%, respectively) (Figure 5C). For both
- treatments, the relative abundance of Proteobacteria was 40.8 and 41.8%, respectively (Figure
- 5A), mainly dominated by Gamma- (32.0 and 23.8%, respectively), Alpha- (0.2 and 11.2%,
- 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 H_2O_2 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 H_2O_2 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).

309 **3.4** *Molecular composition of lampenflora*

A total of 59 different organic compounds were detected from the pyro-chromatograms of the 310 311 samples and their relative abundances were calculated from the peak areas. The compounds were categorized into 5 main product groups with a similar nature or known origin: Alkyl compounds 312 (ALK); aromatic compounds (ARO), polysaccharide-derived (PS), nitrogen compounds (N), and 313 314 contaminants (CONT) (Figure 6, Table S2). All the pyro-chromatograms from the vermiculated surfaces were dominated by polysaccharide-derived substances and alkyl structures. Furthermore, 315 no changes in chemical composition were observed after both H_2O_2 and NaClO treatments. In 316 contrast, clear differences were found for the bare surfaces. Samples treated with H₂O₂ showed 317 the presence of a series of *n*-alkane/alkene doublets, which were not present in the control 318 samples. On the contrary, the application of NaClO resulted in the practically total absence of 319 organic molecules, except for the persistence of a few ALK molecules and several small peaks 320 corresponding to ARO compounds. It is remarkable that the NaClO treatment caused the total 321 322 elimination of PS, which were dominant compounds in all other treatments.

323

324 **3.5** *Thermal analysis*

The total and relative weight loss of the biofilms from both bare and vermiculated surfaces for the different treatments are shown in Table 2. All treated samples (bare and with vermiculations) were characterized by a lower weight loss (2-to-4 times lower) than the control samples. The latter displays a high abundance of very labile organic matter (W1). The differential scanning calorimetry (DSC) data revealed an increase of the thermal resistance of the treated bare biofilms, which is evidenced by the shift of the main exothermic peak to higher temperatures when compared with the control samples. Biofilms with vermiculations showed a greater relative abundance of the most thermally labile fraction (W1) than the corresponding bare sample, which
agreed with the presence of labile organic remains from cyanobacteria and algae depositions and
a decrease of the released heat per unit of organic matter (Q' released).

335

336 **4. Discussion**

Solving the problem of lampenflora in show caves is a challenge and a priority for the 337 338 preservation and sustainable management of these fragile ecosystems. Fine-tuning surface cleaning methods to remove this "green disease", prevent its growth without compromising the 339 340 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 methods (ie, physical cleaning, UVC irradiation, and chemical treatments with NaClO, H₂O₂ or 342 other products), both on lampenflora metabolism and on the treated rock surface composition. 343 Furthermore, there is no standardized action to quantitatively and qualitatively monitor treatment 344 efficacy over time (Baquedano Estévez et al. 2019). The results of this study offer a useful 345 comparison of the processes activated on surfaces by three commonly employed chemical-346 physical methods of lampenflora removal (UVC radiation, NaClO and H₂O₂), shedding light on 347 microbial community evolution following the treatments and potential damage on the substrates. 348 349 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 caves, representing a proxy of the physiological status of the community related to photosynthetic 351 352 activity (Figueroa et al. 2017). The Fv/Fm values recorded on the non-treated green biofilms from the Pertosa-Auletta Cave were in line with those of the lampenflora growing in other caves, 353 such as Cango Cave (South Africa) (mean value 0.74; Grobbelaar 2000) and in La Glacière Cave 354 (France) (mean value 0.70; Pfendler et al. 2017). The chemical treatments (NaClO and H₂O₂) 355

annealed completely the quantum yields already after the first application, due to the oxidation 356 reactions of the organic substrates (Faimon et al. 2003). As revealed by microscopy images, 357 lampenflora was totally removed after NaClO treatment, whereas it was severely damaged when 358 359 using H_2O_2 . In fact, after three months without treatments, the areas treated with H_2O_2 showed signs of lampenflora recovery, whereas those that were cleaned with NaClO remained clean. The 360 visible re-colonization was different for the two chemical treatments: NaClO acted without 361 362 damaging neither bare nor vermiculated surfaces, whereas when H_2O_2 was used, a slight brightening was observed, but also all the death organic matter remained on the surfaces. This 363 latter effect may represent an energy source for the cave biota, as previously described by Mulec 364 365 (2019). Moreover, H₂O₂ treatment was visibly corrosive for vermiculations, mainly composed of calcite (Addesso et al. 2019), producing an effervescent reaction, indicative of oxidation of 366 organic matter and carbonates, producing CO₂ that in turn lead to dissolution phenomena (Trinh 367 et al. 2018). This was already observed by Faimon et al. 2003. Therefore, if on one hand, a higher 368 concentration might be needed to eliminate the organic matter, on the other, this leads to an 369 increase of its corrosive action, requiring a chemical re-equilibration of the solution (which needs 370 to be put in contact with CaCO₃ powder before its application). Mulec (2014) proposed using a 371 carbonate/bicarbonate buffer in the final peroxide solution to remove lampenflora because it 372 373 preserves pH levels not corrosive for cave formations. As previously suggested by Trinh et al. (2018), after a H₂O₂ treatment, a waterjet or brushing would be required for the complete 374 removal of the biomass from the surfaces. Such mechanical treatment would only be feasible on 375 376 hard surfaces like granite (Pozo-Antonio and Sanmartín 2018), and not on soft surfaces, such as those containing vermiculations or pasty moonmilk. 377

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

installed in this section of the cave during the period of observation, without the possibility torestore it again. This confirms that direct light represents the primary driver determining green

biofilm growth (Sanmartín et al. 2012; Piano et al. 2015, 2021).

383 In relation to the taxonomic community composition, the most abundant photosynthetic-based

taxon in the Pertosa-Auletta Cave lampenflora biofilms in the untreated bare surface, was

385 Cyanobacteria, specifically by the aerophytic filamentous cyanobacterial species *Brasilonema*

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-

vacuolated oscillatorioid cyanobacterium, isolated from freshwater (Thu et al. 2012). It is well

389 known that Cyanobacteria are among the pioneering organisms involved in lampenflora

development (Popović et al. 2017; Mulec 2019; Baquedano Estévez et al. 2019; Havlena et al.

2021). The vermiculated untreated surface revealed very low amounts of photoautotrophic

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; Addesso 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 H_2O_2 , 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

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

Despite several studies reporting its efficiency (Borderie et al. 2015; Pfendler et al. 2017), the 408 treatment with UVC lamps, once a month, did not produce alterations, neither in the community 409 410 composition nor in the substrate structure. This could be likely linked to a too mild and ineffective treatment. An increase of time exposition of surfaces from one to four times a month 411 412 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. Analytical pyrolysis was useful to detect shifts in the molecular composition caused by the 414 415 different chemical treatments on the surfaces, highlighting different macromolecular assemblages in the samples. The pyrolysates of untreated bare surfaces were found mainly composed of non-416 specific ARO compounds, commonly observed in natural biomolecules (Miller et al. 2016), PS 417 derived compounds, mainly furan compounds and their derivatives, and minor proportions of 418 ALK, mainly C11 to C21 branched structures with a probable biogenic origin in archaea or algae 419 (Fowler and Douglas 1987; Shiea et al. 1990) and N compounds (mainly pyridine), with a 420 421 probable polypeptide or protein origin (Saiz-Jimenez et al. 2021). The analysis of the chemically treated surfaces provided interesting information mainly about the 422 effects of NaClO on bare surfaces with a sharp reduction in the number of released compounds 423

and a complete absence of the biogenic PS and N compounds, pointing to a high effectivity of

425 this treatment. The effect of H_2O_2 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 polysaccharide429 derived compounds and *n*-alkanes, which are the major organic compounds of cyanobacteria,
430 algae, non-vascular plants and moss.

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

445

446 **5.** Conclusions

The results provided relevant and useful information concerning the efficacy of the most
employed physical (UVC) and chemical (NaClO, H₂O₂) control and removal methods of
lampenflora in show caves. This work offers a comprehensive assessment of biofilm physiology,
chemical composition, as well as of the potential deterioration processes of the underlying rock
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 H ₂ O ₂ treated surfaces showed a recovery of lampenflora after three
456	months without applications, and evident rock dissolution processes activated on surfaces.
457	Indeed, H ₂ O ₂ 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 H_2O_2 , 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.

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481	Declaration of interest statement
482	The authors report there are no competing interests to declare.
483	
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TABLES

a.

Residuals

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.

				596
		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		600
601				
602	b.			
603				
		Df	F value	Pr(>604
	Surface type	1	0.826	0.3695
	Time	9	8.196	4.63 &08
	Treatment	3	168.252	< 2e666

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.

		Bare surface			With vermiculations				
TG		Control	UVC	NaClO	$\mathrm{H}_{2}\mathrm{O}_{2}$	Control	UVC	NaClO	$\mathrm{H}_{2}\mathrm{O}_{2}$
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
Relative Weight Loss (%)									
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

618 FIGURE CAPTIONS

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

623

Figure 2. Maximal PSII photochemical efficiency (Fv/Fm) and percentage of surface covered area by lampenflora, measured before and after the treatments (control, orange; H_2O_2 , green; NaClO, light blue; UVC, violet) on bare rock surfaces (respectively, A and C) and those covered with vermiculations (respectively, B and D). Different letters indicate significant (for α =0.05) differences among treatments over time (small letters) and treatment typologies (capital letters),

630

629

Figure 3. Representative microscopy images of the biofilms on the bare surfaces: without

treatment, control (A-C); treated with UVC irradiation (D-F); treated with NaClO (G-I), and

 $figure{1}{1}$ 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).

according to the Tukey post-hoc tests.

635

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),

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).

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, H ₂ O ₂); 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, $\mathrm{H_2O_2}$ and
647	NaClO treatments. The numbers on the peaks correspond to those listed in Table S2.