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

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Abstract

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 of caves. Photosynthetic-based biofilms usually cause rock biodeterioration and an ecological imbalance in cave ecosystems. In this work, a detailed investigation of the effects of the 3 most commonly used lampenflora cleaning operations (NaClO, H_2O_2 and UVC) was carried out in 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 showed to be corrosive for the rock surfaces covered with vermiculation deposits. The chemical alteration of organic and inorganic compounds by H_2O_2 did not remove biomass, favoring biofilm recovery after three months of treatment. Both NaClO and H_2O_2 treatments were effective at removing photoautotrophs, although the bacterial phyla Proteobacteria and Bacteroidetes as well as Apicomplexa and Cercozoa among the Eukaryotes, were found to be resistant to these treatments. The UVC treatments did not show any noticeable effect on the biofilms.

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

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

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

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

To control and inhibit the "green disease" of show caves, besides acting on the lighting of the cave itself (e.g., duration, intensity, positioning) (Piano et al. 2021), cave managers have applied several physical and chemical cleaning methods to remove the phototrophic biomass from the substrates and prevent further lampenflora development (Mulec and Kosi 2009; Baquedano Estévez et al. 2019). Mechanical eradication using brush and water, liquid nitrogen application, or led lighting systems with emission spectra distinct from lampenflora absorption spectra (Muñoz-Fernández et al. 2021), or even the application of ecologically hazardous chemicals, including herbicides, are the most often methods used against lampenflora proliferation (Mulec 2019; Baquedano Estévez et al. 2019). Among the most commonly cleaning method used today in show caves is the application of commercial bleach (sodium hypochlorite, NaClO). This is a low-cost and efficient method, but expensive in environmental terms by the release of chlorinated 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-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; Baquedano Estévez et al. 2019). Another relatively popular treatment is a germicidal light system with UVC irradiation, applied out of visiting hours. However, a perfect and definitive solution has not yet been found, and the combination of different methods remains the most useful way to control lampenflora (Grobbelaar 2000; Olson 2006; Mulec and Kosi 2009; Cigna 2012; Mulec 2019; Baquedano Estévez et al. 2019; Piano et al. 2021).

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 lampenflora growing on bare rock and on vermiculated surfaces of a karstic limestone cave. 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 underground environments (Addesso et al. 2019, 2020). The techniques used in this study encompassed the application of photochemical efficiency measurements, microscopy, molecular analysis (DNA) and, for the first time, direct analytical pyrolysis and thermogravimetric techniques, which avoid any extraction or manipulation of the samples. A sector along the tourist 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, H_2O_2 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, H_2O_2 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

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 camera (SX620 Canon, Japan) before and after each treatment, for further image analyses. To 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 photosynthesis yield analyzer (MINI-PAM, WALTZ, Germany) equipped with a distance clip 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). These measurements were carried out on 30 minutes dark-adapted surfaces, after covering the surfaces with aluminium foil.

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.

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).

Oven-dried (50 °C) biofilm samples were sputter coated with gold and analyzed using a field 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 resolution images.

2.3 *Images and data analysis*

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

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).

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", "dplyr", "RColorBrewer" and "ggbreak" packages, and using the open-source vector graphics editor Inkscape 0.92.

2.4 *Molecular analysis*

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

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

2.5 *Analytical pyrolysis*

Changes in the molecular chemical structure of rock surfaces were studied by pyrolysis-gas chromatography/mass spectrometry (Py-GC–MS). A double-shot microfurnace pyrolyser (model 2020i, Frontier Laboratories, Fukushima, Japan) attached to a GC-MS Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973 mass selective detector system, was used for the direct analysis of samples. Finely ground samples (c. 10 mg) were placed in small crucible capsules and introduced into a preheated micro-furnace at 400 °C for 1 min. The pyrolysis products were directly injected into the gas chromatograph inlet line heated at 250 °C to prevent condensation. The GC was equipped with a HP-5ms-UI, low polar-fused silica (5%-Phenyl-methylpolysiloxane) (J&W Scientific, Folsom, CA, USA) capillary column of 30 m × 250 μm × 0.25 μm film thickness (Ref. DB-5). Chromatographic conditions were similar to those described in Miller et al. (2022). Compound assignment was achieved by considering diagnostic ions for the main homologous series, via low-resolution MS and via comparison with published 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 ºC) samples were conducted using Discovery series SDT 650 simultaneous DSC/TGA instrument
195 (TA Instruments Inc., New Castle, DE, USA) under a N_2 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 ºC 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 H_2O_2 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 H_2O_2 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 H_2O_2-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 H_2O_2 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 H_2O_2-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

Proteobacteria (59.8%), dominated by the classes Gamma- (24.7%), Beta- (24.7%) and Alpha-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 phylum Cyanobacteria were detected with a relative abundance of 0.4%.

The most abundant phyla of the lampenflora from the bare surfaces treated with UVC were (Figure 5A): Proteobacteria (33.7%), Cyanobacteria (32.0%), Bacteroidetes (11.6%), Firmicutes (6.5%), unclassified Bacteria (5.2%), Acidobacteria (3.0%), Chloroflexi (2.4%), Synergistetes (2.0%) and Actinobacteria (1.1%). The phylum Proteobacteria was dominated by classes Alpha- (14.4%), Delta- (7.7%), Beta- (6.4%) and Gamma-proteobacteria (5.5%), while the phylum Cyanobacteria was dominated by the class Cyanophyceae (32.0%) (Figure 5B) and by the order 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 (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 (5.7%), Chloroflexi (3.3%), Nitrospirae (2.7%), Gemmatimonadetes (2.5%), Firmicutes (1.8%), Actinobacteria (1.6%), and Bacteroidetes (1.0%).

Samples collected from the bare surfaces treated with NaClO and H_2O_2 exhibited similar 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).

Interestingly, the entire bacterial community of the sample from the vermiculated surfaces treated with NaClO was composed of Proteobacteria (100%) phylum (Figure 5A), primarily represented by the Gamma-proteobacteria (99.4%) class (Figure 5B) and by Pseudomonadales (99.4%), at order level (Figure 5C). The vermiculated surfaces treated with H_2O_2 were more biodiverse (Figure 5A), composed of Proteobacteria (46.5%), represented by Alpha- (27.3%), Beta- (8.7%), Gamma- (7.7%), and Delta-proteobacteria (1.8%) classes (Figure 5B), and by Bacteroidetes (34.2%) phylum, dominated by Flavobacteriia (18.1%) and Sphingobacteriia (6.9%) classes, followed by the phyla Firmicutes (5.1%), Verrucomicrobia (3.8%), Actinobacteria (2.5%), and Gemmatimonadetes (1.1%).

Regarding the Eukaryotic community (Figure 5D-F), the controls, as well as the UVC irradiated samples, from bare and vermiculated surfaces, were mostly represented by the phylum Streptophyta (86.3 and 99.0% - 92.8 and 99.3%, respectively for control and UVC treatments) (Figure 5D). This was dominated by Bryopsida class (86.2 and 98.5% - 92.7 and 99.2%, respectively for control and UVC treatments) (Figure 5E) and, at order level, by Pottiales (86.2 and 98.5% - 92.7 and 99.2%, respectively for control and UVC) (Figure 5F). The bare surfaces and those with vermiculations treated with NaClO were mainly characterized by Streptophyta (91.4 and 95.8%, respectively) phylum (Figure 5D), dominated by the classes Magnoliopsida (76.2 and 94.2%, respectively) and Bryopsida (15.1 and 1.3%, respectively) (Figure 5E), and the orders by Lamiales (68.1 and 4.2%, respectively), Pottiales (15.1 and 1.3%, respectively), and Poales (7.6 and 74.3%, respectively) (Figure 4F). The major phyla composing the eukaryotic community of the bare and vermiculated surfaces treated with H_2O_2 were represented by Cercozoa (59.3 and 4.3%, respectively), unclassified Eucaryota (19.0 and 54.6%, respectively), unclassified DNA sequences (13.9 and 5.7%, respectively), Apicomplexa (5.6 and 18.1%, respectively), and Streptophyta (0.6 and 17.1%, respectively) (Figure 5D).

3.4 *Molecular composition of lampenflora*

A total of 59 different organic compounds were detected from the pyro-chromatograms of the 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 (ALK); aromatic compounds (ARO), polysaccharide-derived (PS), nitrogen compounds (N), and contaminants (CONT) (Figure 6, Table S2). All the pyro-chromatograms from the vermiculated surfaces were dominated by polysaccharide-derived substances and alkyl structures. Furthermore, no changes in chemical composition were observed after both H_2O_2 and NaClO treatments. In contrast, clear differences were found for the bare surfaces. Samples treated with H_2O_2 showed the presence of a series of *n*-alkane/alkene doublets, which were not present in the control samples. On the contrary, the application of NaClO resulted in the practically total absence of organic molecules, except for the persistence of a few ALK molecules and several small peaks corresponding to ARO compounds. It is remarkable that the NaClO treatment caused the total elimination of PS, which were dominant compounds in all other treatments.

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).

4. Discussion

Solving the problem of lampenflora in show caves is a challenge and a priority for the preservation and sustainable management of these fragile ecosystems. Fine-tuning surface cleaning methods to remove this "green disease", prevent its growth without compromising the wall integrity and the underground habitat, is desired by most show cave managers (Chiarini et 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, H_2O_2 or other products), both on lampenflora metabolism and on the treated rock surface composition. Furthermore, there is no standardized action to quantitatively and qualitatively monitor treatment efficacy over time (Baquedano Estévez et al. 2019). The results of this study offer a useful comparison of the processes activated on surfaces by three commonly employed chemical-physical methods of lampenflora removal (UVC radiation, NaClO and H_2O_2), shedding light on microbial community evolution following the treatments and potential damage on the substrates. The maximal PSII photochemical efficiency measures (quantum yield) proved to be a valid *in 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 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, such as Cango Cave (South Africa) (mean value 0.74; Grobbelaar 2000) and in La Glacière Cave (France) (mean value 0.70; Pfendler et al. 2017). The chemical treatments (NaClO and H_2O_2)

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 H_2O_2. In fact, after three months without treatments, the areas treated with H_2O_2 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 H_2O_2 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, H_2O_2 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 CO_2 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 $CaCO_3$ 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 H_2O_2 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

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

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 Cyanobacteria, specifically by the aerophytic filamentous cyanobacterial species *Brasilonema angustatum* (39.3%), belonging to the Scytonemataceae family, isolated from the island of Oahu, 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 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 environments, such as Proteobacteria, Nitrospira, Firmicutes, Actinobacteria, and Acidobacteria (Tomczyk-Żak and Zielenkiewicz 2016; Addesso et al. 2020). After the application of both chemical treatments on the bare surfaces, the photoautotrophs were eliminated, but not the bacterial population, mainly Proteobacteria and Bacteroidetes, also present on vermiculated surfaces treated with H_2O_2, whereas those treated with the commercial bleach (NaClO) only showed Proteobacteria. The inefficacy of the chemical treatments in removing non-photosynthetic microorganisms can be related to the presence of microbial species that can tolerate NaClO. In fact, Proteobacteria followed by Bacteroidetes have been identified as the main cause of membrane fouling in membrane bioreactors systems after NaClO backwashing (Wang et al. 2014, 2019). The eukaryotic community from both the untreated surface types was 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 H_2O_2
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 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

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

Concerning thermal analyses, the sharp reduction of the total weight loss of the treated biofilms and the transfer of the relative weight loss from W1 (labile OM) to W3 and W4 for the treated biofilms compared with the control samples pointed out the partial elimination of lampenflora 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 main constituent of extracellular polymeric substances), whereas the thermograms of treated 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 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 DSC for the control at bare surface showed peaks with maxima at 356 ºC, whereas the treated biofilms showed maxima ranging from 404 to 425 ºC. Both peaks fit perfectly with those reported for the combustion of microalgae and fungi, and are attributable to combustion of protein and lipids, respectively (Kang and Yoon 2015).

5. Conclusions

The results provided relevant and useful information concerning the efficacy of the most employed physical (UVC) and chemical (NaClO, H_2O_2) 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

the most efficient method in relation to both surface sterilization and visible cleaning over long time, with unaltered underlying rock substrates. However, the toxicity of chlorine compounds is known and an important drawback, requiring the use of diluted solutions, thus limiting the efficacy of this method. The H_2O_2 treated surfaces showed a recovery of lampenflora after three months without applications, and evident rock dissolution processes activated on surfaces. Indeed, H_2O_2 treatment promoted the release of alkyl chemical structures and a visible deterioration of vermiculation deposits. In addition, the organic matter was not eliminated by the application of H_2O_2, and consequently the remaining organic matrix would need to be removed through brushing or water jets in order to avoid undesirable effects on the ecological balance of the caves. Based on the conditions tested in this work, the UVC irradiation treatment was not an effective method for the removal of lampenflora.

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Declaration of interest statement

The authors report there are no competing interests to declare.

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TABLES

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

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		

b.

	Df	F value	Pr(>F)
Surface type	1	0.826	0.367
Time	9	8.196	4.63e-08
Treatment	3	168.252	< 2e-16
Residuals	66		

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

		Bare surface				With vermiculations			
TG		Control	UVC	NaClO	H_2O_2	Control	UVC	NaClO	H_2O_2
Moisture and very labile OM-W1	50–120 ºC	2.1	0.4	0.3	0.3	2.8	0.9	0.4	0.4
Labile OM-W2	120–200 ºC	0.7	0.2	0.2	0.1	0.9	0.4	0.1	0.2
Int OM-W3	200–400 ºC	2.1	0.8	0.7	0.6	2.2	1.3	0.5	0.7
Recalcitrant OM-W4	400–600 ºC	3.1	1.0	1.1	0.7	3.6	1.4	1.5	0.8
Total weight loss	50–650 ºC	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 ºC	26	16	13	18	29	22	16	21
Labile OM-W2	120–200 ºC	9	8	8	6	9	9	4	9
Int OM-W3	200–400 ºC	27	35	31	34	24	33	20	32
Recalcitrant OM-W4	400–600 ºC	39	41	48	42	38	36	60	38

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.

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), according to the Tukey post-hoc tests.

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 treated with H_2O_2 (J-L). The corrosion features as well as the collapsed biological masses due to the H_2O_2 treatments are indicated with arrows (**L**).

Figure 4. Representative microscopy images of the biofilms on the surfaces with vermiculations: 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 due to the H_2O_2 treatments are indicated with arrows (**L**).

Figure 5. Bacteria and Eukaryotes composition of the lampenflora from bare (A-C) and vermiculated (D-F) surfaces for each treatment type (control, UVC, NaClO, H_2O_2); the barplots show the relative abundances (%) at phylum (A,D), class (B,E), and order (C,F) levels.

Figure 6. A. Representative annotated pyrograms (Py-GC/MS). B. Relative abundance (%) by compound group in the biofilms from bare and vermiculated surfaces for control, H_2O_2 and NaClO treatments. The numbers on the peaks correspond to those listed in Table S2.