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RESEARCH ARTICLE

UV-C as a means to combat biofilm proliferation on prehistoric paintings: evidence from laboratory experiments

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Abstract A laboratory investigation of UV-C effects was conducted over a 62-h period: a much higher dose than in classic UV-C treatment was applied to five pigments and two painting binders used by prehistoric humans. Colorimetric parameters were compared to a control to see if UV-C can change pigment and binder color. Infrared spectroscopy, scanning electron microscopy, inductively coupled plasma and X-ray crystallography were also carried out to confirm colorimetric measurement. In order to understand how microorganism may physically deteriorate paintings, limestone blocks were painted and monitored until their complete colonization by algae, cyanobacteria, fungi and/or mosses. The results show that UV-C has no effect on mineral compounds. Conversely, it is noteworthy that binder color changed under both UV-C light conditions as well as in visible light. Concerning painted blocks, a fast proliferation has been observed with deterioration of the paintings. These results show the high importance of treating biofilm as soon as possible. Moreover, these findings may be a promising avenue inducing cave managers to use friendly UV-C light to treat contaminated cave paintings and also in the prevention of biodeterioration by lampenflora.

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Introduction

Subterranean sites have long interested both the academic world and the general public, with paleo-anthropological and archaeological discoveries, especially of prehistoric paintings, contributing heavily to their fascination. This prehistoric rock art from the upper Paleolithic between −40,000 and −10,000 BC (Chalmin 2003) constitutes the most unique feature of these sites, revealing a story of human occupation and activity in one of the world's most challenging environments.

Prehistoric cave art, along with speleothem formations, make these places major tourist attractions, though causing increases in temperature and carbon dioxide. Added to the use of high intensity artificial lighting used for tourist visits (Falasco et al. 2014; Borderie et al. 2015), these emerging environmental conditions promote the development of photosynthetic microorganisms such as algae, cyanobacteria (Cennamo et al. 2012; Popović et al. 2015; Borderie et al. 2016) and fungi (Saiz-Jimenez et al. 2012) which in turn make organic matter and nitrogen bioavailable to heterotrophic fungi and bacteria (Hauer et al. 2015; Urzì et al. 2016).

The resulting photosynthetic biofilms thus become a major problem for curators causing not only esthetic problems (Ciferri 1999; Cutler et al. 2013a; Adhikary et al. 2015) but also, more dramatically, a deterioration of parietal paintings (Cuzman et al. 2010, Cutler et al. 2013b; Adhikary et al. 2015) through the production of organic acid (Mulec 2005; McNamara et al. 2006; Albertano 2012) or, mechanically, by growth within microfissures, which gain in volume provoking increased cracks in the paintings (Danin and Caneva 1990, Cennamo et al. 2016). For example, in the world famous

ancient wall paintings of the Lascaux Cave (France), the development of algae on artworks estimated to be 17,300 years old led the authorities to an abrupt closure of the cave in the 1960s to limit the threat (Bastian et al. 2010). Moreover, the ceiling, walls and sediments were colonized by the fungus *Fusarium solani* in 2001 (Bastian et al. 2010; Martin-Sanchez et al. 2012).

Nowadays, cave managers use treatments such as high-pressure cleaners, bleach, or chemical compounds, which may degrade cave walls and mineral structures (Faimon et al. 2003; Mulec and Kosi 2009). In addition to being retentive and harmful to the environment, with a risk of contaminating the groundwater, their efficiencies are variable.

In order to protect natural heritage sites, new friendly environmental treatments have been investigated, especially the use of UV-C light (Borderie et al. 2014) which has shown itself to be efficient in the air and water treatment industries, but also in hospitals as a method of sterilization. UV-C light is also effective against algae and cyanobacteria in the laboratory and shows promising results in touristic caves (Borderie et al. 2011; Borderie et al. 2014). However, the potential effect of UV-C on parietal paintings and limestone cave walls has been poorly addressed due to (1) the nature of pigments: prehistoric paints were made with natural pigments such as ochre, coal and manganese dioxide (Bikiaris et al. 1999; Arocena et al. 2008; Darchuk et al. 2011; Bonneau et al. 2012) and (2) the complexity of the binders that hold the pigments in the paint: certain organic compounds including blood, beeswax, animal fat and vegetable oils (Arocena et al. 2008; Prinsloo et al. 2008; Lofrumento et al. 2012).

We therefore investigated the effects of UV-C radiation on prehistoric pigments and binders. To determine whether some molecular or crystalline modifications appear after irradiation, measurements were taken with Fourier transform infrared spectroscopy (FTIR) and X-ray crystallography (XRD). Moreover, inductively coupled plasma mass spectrometry (ICP) and scanning electron microscopy (SEM-EDX) were used to compare elemental pigment composition. FTIR, SEM-EDX and XRD methods have been previously used to study pigments and binders in prehistoric paintings (Zoppi et al. 2002; Mortimore et al. 2004). To monitor possible color changes, colorimetric parameters were assessed.

Materials and methods

Effect of microorganism growth on paintings

To ensure that microorganisms might grow on each pigment used in the composition of prehistoric paintings, but also to understand biofilm development, 2 g of pigments was mixed with 20 ml of water. The mixture was painted onto limestone blocks. To simulate biofilm colonization in laboratory, blocks

were placed on vermiculite to keep them moist but no inoculation has been carried out. The temperature (15 °C), moisture (100%) and light (150 μmol m^{-2} s^{-1}, 16 h per day) gave favorable conditions for microbial colonization by both photosynthetic and non-photosynthetic microorganisms. Seventy-three days after the beginning of the experiment, blocks were no longer moistened for 1 week so as to simulate cave dry periods.

Preparation and irradiation of paints and binders

In this study, we tested five pigments and two binders used by prehistoric humans in caves (Fig. 1): coal, bone char, manganese dioxide, red ochre, yellow iron oxide, beeswax and lard. Colorimetric parameters, XRD, ICP, SEM-EDS and FTIS analysis were carried out on 8 g of each pigment and binder (Figs. 2, 3 and 4). Pigments powder and binders were placed in glass Petri dishes (5 cm diameter). Samples were irradiated for 62 h with 8 UV-C lamps (Philips, 25 W each = 200 W, λ max = 254 nm) corresponding to a dose of around 4800 kJ m^{-2}. Several measurements were taken prior to treatment and 10 days after. During this 10-day time lapse, samples were stored under light conditions (150 μmol m^{-2} s^{-1}) 16 h per day.

### Colorimetric measurement of pigments and binders

Dry pigments and binders were used in order to avoid color change due to moisture. Colorimetric parameters were measured with a spectrophotometer (CM-600d KONICA MINOLTA, illuminant D65, SCI mode and 8 mm diameter target mask). Color measurements were analyzed according to the CIELAB color system. The dark-light scale (L*) is associated with the lightness of the color and ranges from bottom (value 0, black) to top (value 100, white). The a* and b* scales are associated with changes in redness-greenness (positive a* is red and negative a* is green) and changes in yellowness-blueness (positive b* is yellow and negative b* is blue) (Borderie et al. 2014).

Fourier transform infrared spectroscopy

After grinding in a mortar about 2% by weight of each pigment in anhydrous KBr, the mixture was placed in a mechanic press to obtain a thin and translucent pastille. Then the spectra were measured with a Fourier transform infrared spectrometer (IRAffinity-1, Shimadzu) at room temperature.

Infrared spectra were measured with the Perkin-Elmer Spectrum 100–400 spectrometer. The samples were placed in the ATR bench diamond platinum (Single Reflexion Attenuated Total Reflectance) and smashed with a press, previously washed with ultrapure ethanol. In order to make a comparative study, a number of scans (30) was used for all samples.

Fig. 1 Growth of algae, fungi, and mosses on a limestone block previously painted with red ochre
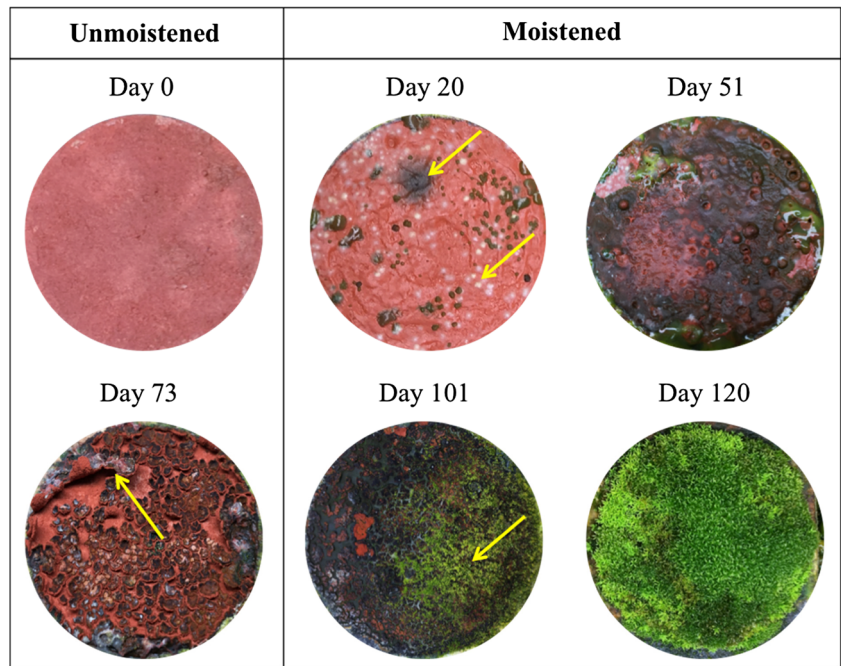

X-ray crystallography

Pigment samples were crushed in a mortar with an agate pestle and placed in a mined glass slide. The diffractometer used was an automated Bruker D8 AdvanceLynxEye Detector (cobalt tube λ Kα1 ≈ 1789 Å). The scan was 0 to 70° 2θ, with a speed of about 0.005° per second. The diffractograms obtained were traced on software Diffrac Plus Measure and interpreted using Diffrac Plus Eva.
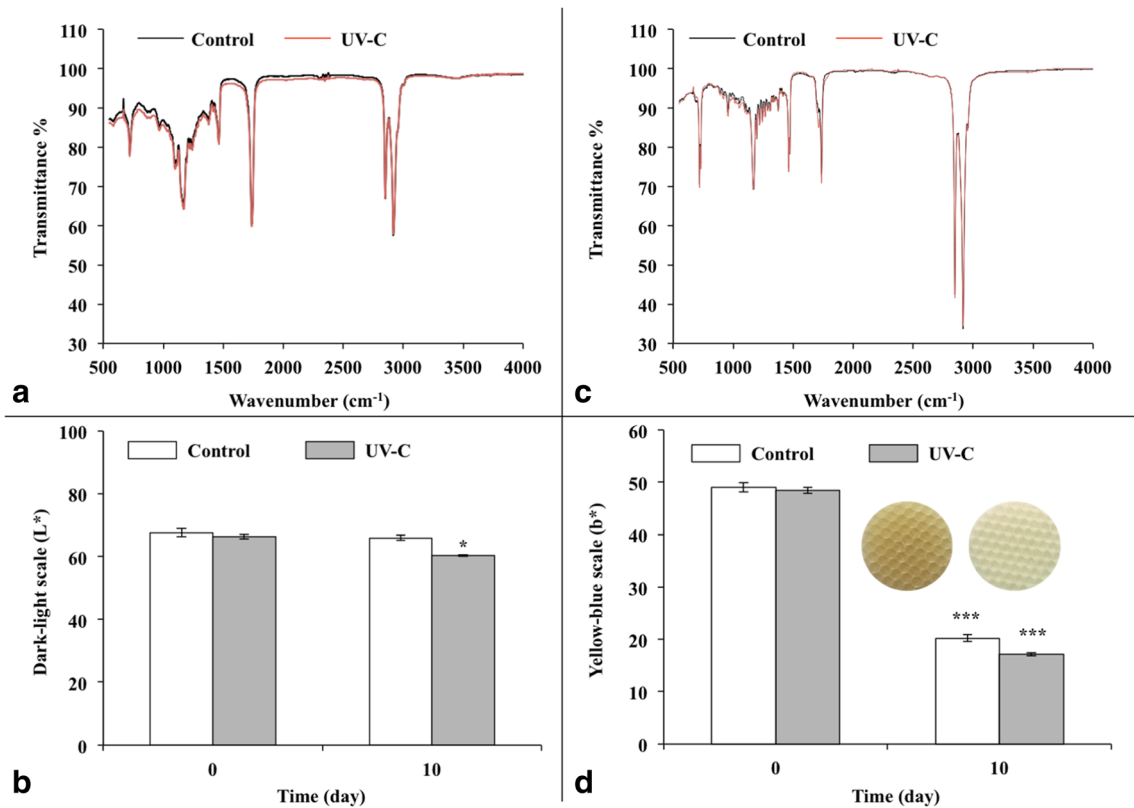
Fig. 2 Infra-red analysis of treated and untreated lard (**a**) and beeswax (**c**). Graphics **b** (lard) and **d** (beeswax) show results of colorimetric measurements of both treatments
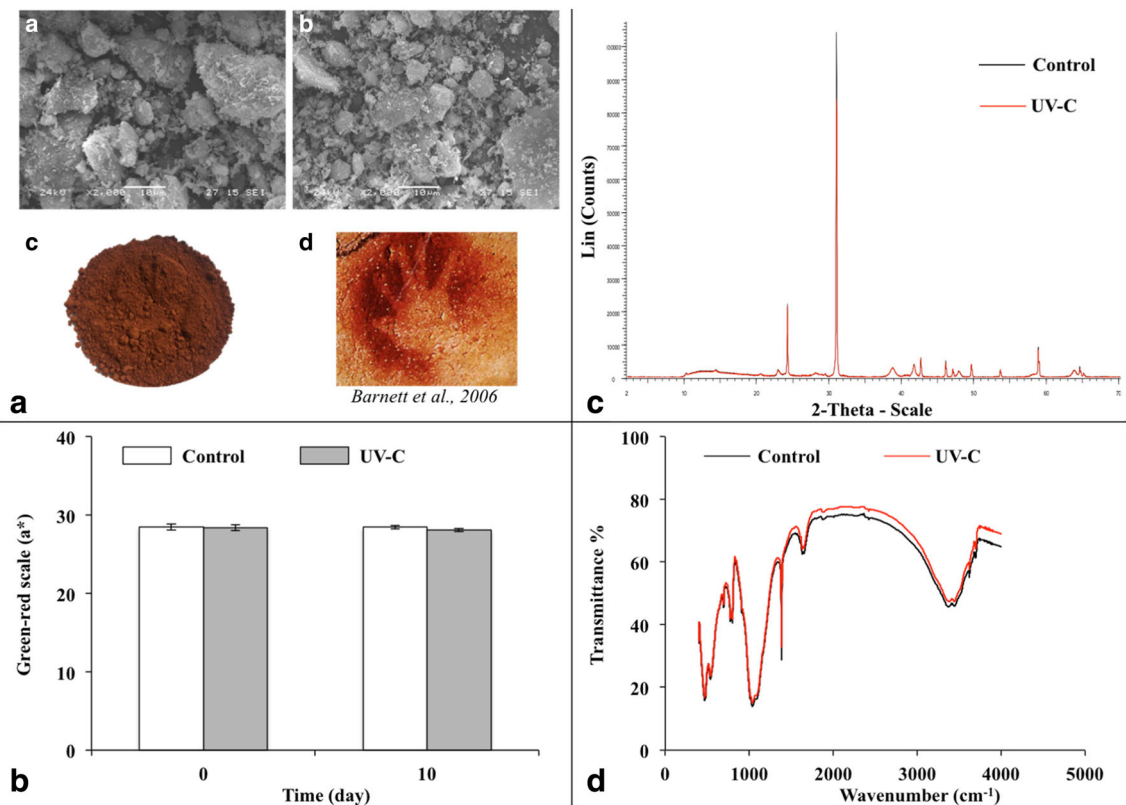
Fig. 3 a SEM picture of red ochre before (*A*) and after (*B*) UV-C radiations. The red powder (*C*) has been used by prehistoric human (*D*). Colorimetric measurements (**b**), DRX analysis (**c**), and infrared spectrometry (**d**) have been carried out before and after UV-C treatment

Inductively coupled plasma mass spectrometry (ICP)

Chemical components in pigments were determined before and after UV-C treatment. Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Se, Si, Sn, Sr, Ti and Zn were measured using ICP-AES after acid mineralization (1 ml HNO$_3$ and 2.5 ml HCl) of 0.25 mg in Digiprep Mineralizator. After adding ultra-pure water to 25 ml, samples were filtered (1 μl). To assess the analytical quality, a standard reference material (Loamy Clay) was subjected to the same protocol. Chemical element concentrations are reported in Table 3.

Scanning electron microscopy equipped with X-ray microanalysis (SEM-EDX)

The measurements were carried out by a JEOL 5510 microscope coupled to an SamX 30 mm^2 EDX module and equipped with a thermal emission gun. It was used for both imaging and EDS analysis, in high vacuum mode with an acceleration voltage of 24 kV, a spot size of 30 and a working distance of 21 mm. The powder is fixed on analytical carbon scotch and then covered with 20 nm of carbon by thermal sublimation.

Statistical analysis

All statistical analyses were performed using R.2.14 software at the significant level of 0.05. An ANOVA statistic test was used for colorimetric measurements. All experiments were conducted with four replicates.

Results

Microorganism growth on paintings

Red ochre painting blocks maintained under culture conditions showed rapid colonization by micro-algae (predominance of unicellular green alga *Bracteacoccus* sp.), cyanobacteria (*Phormidium* sp.) and fungi (*Verticillium* sp.) (Fig. 1). After 2 weeks, these different microorganism species formed colonies. Fifty-one days after the beginning of the experiments micro-algae and cyanobacteria invaded the entire painted block area. At day 73 (Fig. 1), dry colonies cracked and flaked from the block taking away a part of the pigment. After 3 months, mosses began to grow and by the fourth month had completely invaded the painted limestone block. These mosses were identified as *Bryum argenteum*, *Bryum*

Fig. 4 X-ray microanalysis on red ochre **a** before and **b** after UV-C radiations
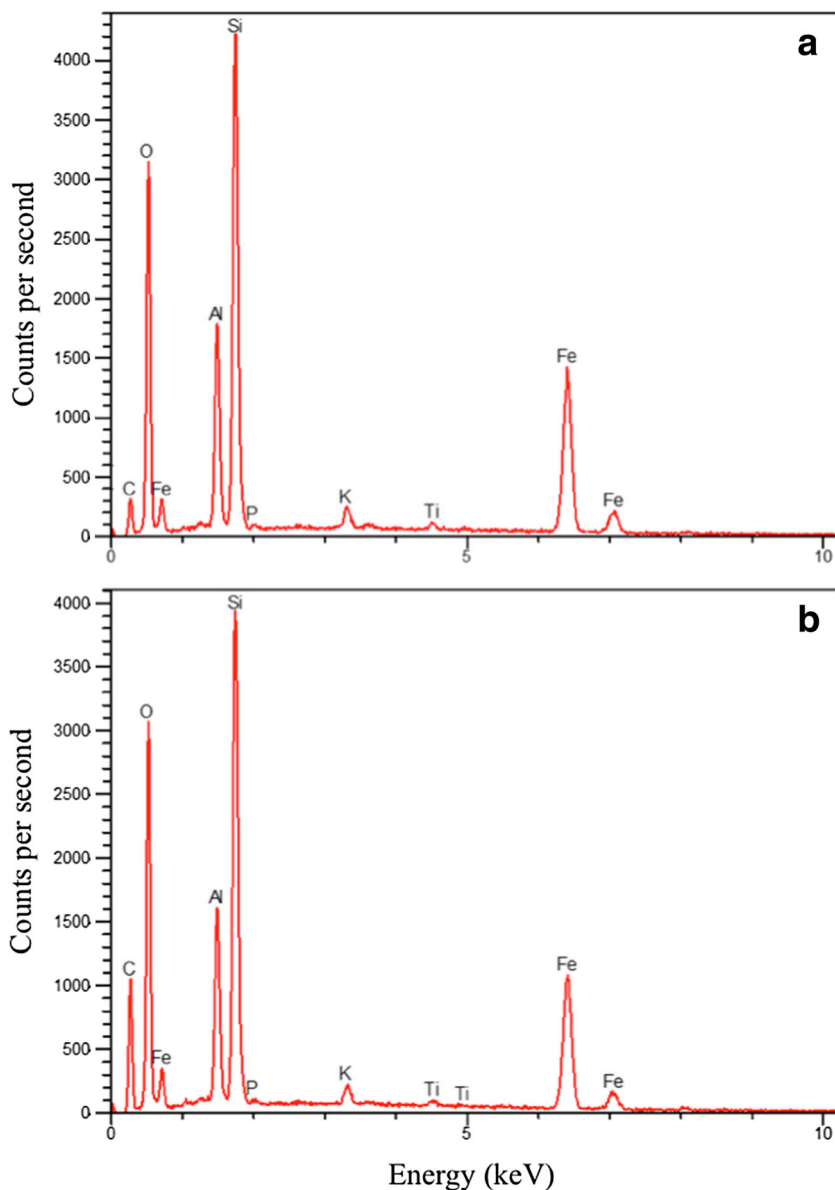
capillare and *Barbula convoluta* with the help of the "Mosses and Liverworts of Britain and Ireland" guidebook.

Monitoring of colorimetric parameters of pigments and binders

Pigments and binders were monitored with one of the CIELAB parameters, depending on their color (Table 1).

Colorimetric results show that the binder color changes after exposition to UV-C light. The lard color became significantly darker (L*) after radiation (Fig. 2b), but no change was observed for the control (Table 1), while the beeswax showed a significant decrease in the yellow color (b*) for both the control and UV-C irradiated samples (Fig. 2d).

As for pigment parameters, these were not statistically different in comparison to the control (Table 1). Figure 2a shows

a* parameters of red ochre. The data of the four other pigments are not shown, but the same non-significant difference between control and treated samples also appears.

Fourier transform infrared spectroscopy

The study by infrared spectroscopy was realized to verify the effect of UV-C on molecules composing pigments and binders. The FTIR spectra obtained for all pigments show that all peaks, which were present before irradiation to UV-C, are present after (Fig. 3d). The only difference is around 2300–2400 cm^{-1} and this is due to the CO_2 present in the air and should not be taken into consideration. Results obtained for binders are similar: the curves can be superimposed and conserve all peaks before and after UV-C treatment (Fig. 2a, c).

Table 1 Statistical results (*p* value) of colorimetric measurements

Treatments	Red ocher	Yellow iron	Manganese dioxide	Bone char	Coal	Beeswax	Lard
Control	0.39	0.27	0.70	0.10	0.69	<0.05	0.32
UV-C	0.27	0.76	0.99	0.11	0.39	<0.05	<0.05
Colorimetric measurement	a*	b*	L*	L*	L*	b*	L*

*a** green-red scale, *b** blue-yellow scale, *L** dark-light scale; *p* value was obtained with an ANOVA test

X-ray crystallography

X-ray crystallography revealed the crystalline composition of the pigments (Table 2) before and after UV-irradiation. Like infrared analysis, the X-ray crystallography graphics obtained for both UV-C exposed pigments and the control show no change in the characteristic peaks (Fig. 3c). Certain peaks corresponding to mineral elements sometimes appeared higher in the control than in the treated powder, a change attributable to a difference in concentrations. Results from UV-C treated pigments showed the same peaks in the same place with no peaks disappearing nor appearing. This suggests that no product has been degraded nor any by-product formed.

ICP-MS

The five pigments were analyzed with ICP before and after UV-C irradiation to determine chemical element concentration. Results, reported in Table 3, showed that Fe was the element, which differed the most between both treated and untreated samples (−0.8%). However, this low difference was not significant.

Scanning electron microscopy equipped with X-ray microanalysis

UV-C effect on pigments was tested by SEM analysis. Pictures of UV-C treated pigments showed no difference in mineral structure with the control (Fig. 3a). This result is corroborated by X-ray microanalysis (Fig. 4). For instance, concentration in O, Si, Fe, Al, K, Ti and P, as quantified in red ochre control, did not change significantly as compared with the treated pigment. The highest difference was observed for oxygen (+2.7%) and the lowest for phosphorus and titanium (+0.04 and −0.03%, respectively).

Discussion

Dynamics of microorganisms on painted limestone blocks

The results show that the assembly of microorganisms started with the proliferation of the pioneer unicellular green alga *Bracteacoccus* sp. This result is intriguing since a similar trend with massive development of *Bracteacoccus* sp. (Lefèvre 1974) after 12 years of tourist visits was also reported in the world's most famous cave, the Lascaux Cave (Dordogne, France). This colonization resulted in a threatening of the integrity of the unique and famous 17,000-year-old prehistoric paintings and ultimately leading to the cave being closed to the public in 1963. This strain may have originated from outside, via vectors such as visitors, air flows or some animals able to acclimate to low light and carbon dioxide conditions (Cigna and Burri 2000). Another pioneer unicellular green alga identified as *Chlorella minutissima* (Aleya et al. 2011; Borderie et al. 2014) proliferated in the Moidons Caves giving rise to biofilm formation. The same occurred on our blocks as, over time, they became greenish due to the presence of organic pigments such as chlorophyll, giving rise to biofilm formation with the development of algae and cyanobacteria after only 2 weeks (Fig. 1). In this study, mosses, too, are capable of totally invading the paintings after 4 months. From a theoretical ecologist's perspective, our experiment simulates habitat-specific selectivity and random dispersal in building multi-specific assemblages (Hubbell 2001; Bell 2005). It becomes obvious that to counteract the 'green' disease responsible for aesthetic damage (Fig. 1) and biodegradation (Ciferri 1999; Cuzman et al. 2010; Adhikary et al. 2015), it is urgent to find an environmentally friendly treatment, one that will eradicate the pioneer unicellular green alga that induces biofilm formation on prehistoric paintings. Finally, further molecular studies like fingerprinting (Cennamo et al. 2013) or sequencing (Cennamo et al. 2012) should be addressed to understand the colonization kinetics of all organisms growing on pigments of heritage monuments.

Monitoring of colorimetric parameters of pigments and binders

The initial analysis undertaken in this study was via the monitoring of the colorimetric parameters of pigments and binders before and after UV-C treatment. In contrast to Athanassiou et al. (2000), we observed no change in any pigment; those authors, however, reported that the use of a UV-C laser caused a darkening of the pigment and an alteration in molecular composition proved by X-ray diffractometry. Chappé et al. (2003) found the same results with the use of a UV-C laser on medieval pigments (zinc white, red and white lead, brown

Table 2 Mineral composition of the five studied pigments

Pigment	Manganese dioxide	Bone char	Red ocher	Yellow iron oxide	Coal
Composition	Hematite Jacobite	Quartz Hydroxyapatite	Quartz Kaolinite Graphite	Quartz Hematite Kaolinite Illite Goethite	Graphite

ochre, cobalt blue). Finally, Castillejo et al. (2002) demonstrated that direct laser irradiation (248 nm) induces various degrees of discoloration that depend strongly on the nature of the pigment. This effect takes place mainly on the surface layer of the sample (Castillejo et al. 2002). Furthermore, laser use is known to induce thermal, mechanical, thermochemical and photochemical effects (Luk'yanchuk 2002) to pigments, which we confirmed in this study.

Binder colorimetric parameters tested in this study showed interesting results. Both lard and beeswax show a decrease in color parameters of L* and b*, respectively (Fig. 1d). These results suggest the deleterious effect of UV light on organic compounds. In fact, UV-C can alter biological molecules, such as keratin, collagen and cellulose (Caneva et al. 2008). However, the beeswax control samples ($n = 4$) show the same color change as the irradiated samples, meaning that, independently of light source, beeswax and especially carotenoid, responsible for the yellow color, are light sensitive. Nevertheless, and interestingly, infrared results show no differences between treated and non-treated samples. The color change observed on beeswax and lard is new and comprises the only argument against the use of UV-light. However, the presence of organic matter in prehistoric paintings is controversial (Chalmin 2003). We infer that organic compounds are not stable over several tens of thousands years. Pallipurath et al. (2015) also show that binders consisting of fats are not stable. Moreover, Couraud (1988) reported that presence of binders could not permit a good painting conservation and make them sensible to fungi. In fact, heterotrophic microorganisms might use these sources of organic matter for their own growth (Cennamo et al. 2016). It must also be emphasized that even in the absence of light, the binders polymerize over centuries leading to a slow change from organic to inorganic.

Concerning X-ray crystallography, infrared spectroscopy, ICP and scanning electron microscopy experiments, a change in the mineral components contained in pigments has been asserted when only one among the four techniques was used, never when these are used simultaneously. Colorimetric analyses were also essential since alteration may occur within a monolayer crystal surface and might be not apparent in the various spectra, except in colorimetry (Luk'yanchuk 2002). This was expected since, theoretically, the UV-C photon energy is not powerful enough to break mineral-chemical bonds. These results were also corroborated by those of Gibeaux et al. (2014) and Borderie (2014), who conducted this experiment with lower UV-C intensities and time periods (16 h at 45 W maximum, and 13 h at 100 W, respectively). In addition, Rifkin et al. (2015) showed that the red ochre, which has been used as a coloring agent in Africa for over 200,000 years and is still used by women of the Himba ethnic group in Namibia (Mcbrearty and Brooks 2000), inhibits the harmful effect of UV-radiations on their skin. In fact, red ochre absorbs, reflects or scatters UV-radiation (Rifkin et al. 2015).

Conclusion

Our approach shows that UV-C light can potentially be used as an environmentally friendly alternative to chemicals in order to combat biofilm contamination of prehistoric paintings. Microorganisms are killed by radiation while no change in pigment color nor in their chemical structure has been observed. Change in beeswax has been observed under UV-C light but also under VIS-light. However, many authors reported that organic matters are not stable in the time and that no binders could be detected in paintings. Our recent study showed that UV-C (553 KJ m^{-2}) could be used with high efficiency against biofilms in tourist caves without prehistoric paintings and without recolonization during 2 years. Further studies must be conducted on some small part of original paintings to check definitively the safe use of UV-C on mineral pigments.

Table 3 ICP results of red ochre pigment

Chemical elements		Al	Ca	Fe	K	Mg	Na	P	S	Si	Ti	Zn
Concentration (%)	Control	27.1	0.5	66.9	2.9	0.5	0.3	1.2	0.1	0.1	0.1	0.1
	UV-C	27.7	0.5	66.1	3.2	0.4	0.3	1.1	0.1	0.1	0.1	0.1

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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