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Innovative solutions for prehistoric paintings - atmospheric pressure plasma and phosphate consolidant for the preservation of the Magura Cave (Bulgaria)

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Abstract. The present study tackles the problem of a sustainable and efficient conservation of cave art, by using innovative materials and techniques for the different steps of the restoration process - Biodeactivation, Biocolonization prevention and Consolidation. The Magura cave in northwest Bulgaria is the case study. It contains an impressive display of prehistoric paintings made of guano as far back as 5'500 years ago. In the last forty years the cave suffered progressive microbial colonization. The detrimental effects are biofilm formation, physical penetration into the stone and chemical reaction with the stone/paintings by pigments. Therefore, as a first step, we investigated biodeactivation by non-thermal plasma sterilization. The oxidative atmosphere obtained introducing Ar/O_2 (0.2 and 0.1) in the plasma device, was carried out on lab samples inoculated with the targeted for Magura Cave microorganisms. The main advantage of the non-contact treatment with atmospheric pressure plasma (APP) is the lack of any mechanical and chemical modification of the underlying stone/guano layers. As for sterilization of wounds on human skin the plasma treatment on wet surfaces produces mainly hydrogen peroxide and nitrates which lead to a localized reduction of the pH. The obtained biodeactivation is assured without heat (< 40 °C), toxic and environmentally harmful liquid. In a second step, we tested two possible alternatives for consolidation of the cave. A commercial ethyl silicate (ES) product was compared with an innovative phosphate treatment, based on application of a hydro-alcoholic solution of a phosphate salt (diammonium hydrogen phosphate, DAP). The consolidation efficiency and compatibility of the ES and DAP consolidants were investigated on samples representative for the Magura Cave substrate, i.e. stone alone and stone covered with guano to resemble the prehistoric drawings. In addition, a combination of plasma activation of the stone surface and consolidation was tested, to investigate whether the two treatments may have a synergistic effect, thus making the combined treatment more efficient than consolidation alone.

1. Introduction

The Magura cave is a splendid combination of natural and human art offering a rich collection of geological formations (stalactites, stalagmites, columns, cave pearls, flows of moonmilk) and a Gallery with prehistoric paintings made of guano as far back as 5'500 years ago.

As soon as the cave was opened to the public in 1961, it attracted many visitors and the microbiological balance was changed. In the last ten years the parameters of the Gallery with paintings have shown sharp variations of temperature/humidity and rich nutrients in the guano. The temperatures range from 22-25 C during the opened for public hours to 12-13 C at night and humidity is in the range 89-65%. These high values are favourable environmental conditions for many different types of microorganisms. Some of the permanent components of the microbial community formed in the Gallery are Penicillium spp., Aspergillus spp., Trichosporon spp., Torulaspora spp., Proteobacteria, Acidobacteria, Actinobacteria [1]. All of them have been demonstrated as the causative agents in deterioration of stone [2]. Inappropriate artificial illumination of archaeological remains and their interior works of art resulted in the uncontrolled development of photosynthetic microorganisms, primarily cyanobacteria and microalgae [3], forming greenish biofilms that contribute to surface deterioration. Fungi such as Aspergillus spp and Penicillium spp. - typical for the Magura Cave and often found in Lascaux Cave, are known to solubilise and mineralize phosphorus from inorganic and organic pools [4][5].

The rapid extension of the fungal and bacterial colonies in combination with the natural erosion led to detrimental biogeochemical/physical and aesthetic effects in the Gallery with the paintings (Figure **1**). The limestone shows powdering, blistering and fragmentation. Green, white and black stains cover some of the paintings and stone surface, compromising also the aesthetic perception of the cultural monument. The condition led to the closure of the Gallery with the paintings in 2019 and provoked urgent need of multidisciplinary research and strategy for preservation.

A combination of low humidity and low temperature is the simplest way to control microbial growth [6], but the cave is home of 8 different bat species and the environmental control is impractical. Regular cleaning and application of biocides has become a routine practice in the conservation of cultural heritage materials. However, biocides are a difficult tool for preservation. Many are too caustic for environmental use or are not strong enough to discourage microbial growth [7]. Frequently several chemicals need to be combined but the effective eradication is limited in time.

Among the conventional techniques, our research efforts have led to the evaluation of an alternative method for the deactivation of micro-organisms by applying non-thermal plasma. Biodeactivation by atmospheric pressure plasma (APP) appears to be a promising technique to eliminate organic material from surfaces without having toxic residue effects and without damaging the underlying surface [8]. Despite its success in treatment, the application on rock art is still a new research area. Therefore, our study focuses on the comparison between APP and two traditional biocides.

To deal with the specific environmental conditions in the Gallery with the paintings, we need also to artificially consolidate the disintegrated material and to preserve the stone from biological and chemical weathering. Hence, as a second step, we tested two possible alternatives for consolidation of the stone. Failure of current conservation products is evident all over Europe: organic polymers have clearly demonstrated to be not compatible with the stone and wall painting surfaces, undergoing photo-oxidative modifications reducing their conservative properties and hindering their removal too. For this reason, in this study two alternative inorganic consolidants were tested: a commercial product based on ethyl silicate (ES), enhanced with anti-microbial capacity, and an innovative treatment based on diammonium hydrogen phosphate (DAP)[9][10].

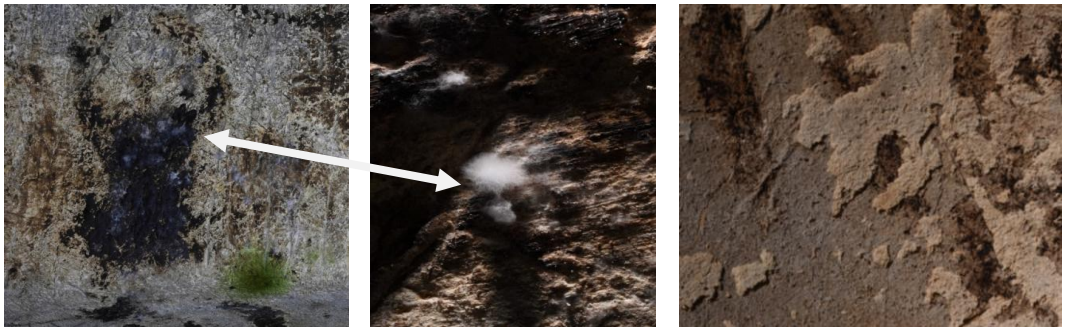


Figure 1:Details of the guano drawings in the Magura cave: microbial colonization and stone weathering

2. Materials and methods

2.1. Biodeactivation and Biocolonization prevention

2.1.1. Samples. In order to enable a better microscopic imaging and simplify the evaluation of the biofilm deactivation, first treatments were carried out on inoculated glass samples (1×1 cm). Laboratory strains taxonomically related to the both genus Penicillium and Aspergillus were used (Laboratory of Geological Microbiology - Department of Microbiology, Sofia University St. Kliment Ohridski, Bulgaria and Ca' Foscari University of Venice, Italy). The culture medium used for reactivation was solid YGC (Oxoid®). The micro-organism suspension was incubated aerobically at 26°C for 72 hours. In controlled aseptic conditions (bacteriological box), prints were prepared on sterile glasses. The latter were transferred to sterile plates. As a second step, the treatments demonstrated best biofilm deactivation were repeated on laboratory performed samples representative for the Gallery with the paintings: limestone inoculated with Penicillium spp./Aspergillus spp. (5×5×2 cm) and guano on limestone inoculated with Penicillium spp./Aspergillus spp.(5×5×2 cm).

2.1.2. Biodeactivation methods. To establish the most effective biodeactivation method the potential of **APP** was compared with that of two traditionally used biocides **Biotin T (3 % in ethanol)** and **Preventol RI 80 (5 % in deionised water)**. All biocides were purchased from CTSsrl. The effectiveness of biodeactivation with APP was tested using two different commercial atmospheric pressure plasma jets: **kINPen** (Neoplas GmbH, Germany) and **Stylus Plasma Noble** (Nadir Srl, Italy). With kINpen the treatments were performed at 1mm working distance,8 W power consumption, 230 V, 50/60 Hz power supply, inlet pressure 1.5 bar, **Ar/O_2 (98.8/0.2%)** as working gas at 5.5 L/min gas flow. With Stylus Plasma applied parameters were: 3mm working distance, 5 W power at 16 KHz/30 W power at 27 MHz, **Ar/O_2 (99.9/0.1%)** as working gas at 7.3 L/min gas flow. In addition, a combination of traditional biocide in a very low concentration and plasma sterilization was tested (**Preventol RI 80 1%(in deionised water) + APP kINPen (Ar/O_2 (99.8/0.2%))**.

2.1.3. Treatments. Biotin T, a compound based on n-octyl-isothiazolone and a quaternary ammonium salt, was applied as a 3 % solution in ethanol. Preventol RI 80 - liquid formulation of benzalkonium chloride, was used as 5 % solution in deionised water. Both materials were applied as per the manufacturer's instructions with a paintbrush until the entire surface was thoroughly saturated with the biocide solution. For the biodeactivation by plasma sterilization the oxidative atmosphere obtained introducing Ar/O_2 0.2% (kINPen) and Ar/O_2 0.1% (Stylus Plasma**)** in the plasma device was applied. Normally, the stone surface in the Gallery with the paintings is wet, due to the high values of humidity (89 %). Therefore, the plasma performance was evaluated on wet inoculated surface (a drop of deionised water was dripped onto the surface just before plasma treatment). The biofilm samples were treated for 60, 120 and 300 s. The control samples were exposed to the gas flow without plasma ignition. Before plasma treatment each sample was washed for several minutes using distilled water

and dried by air flow to ensure that the substrate is covered with an adherent, nonwashable biofilm. The combined method biocide+plasma was performed as applying of Preventol RI 80 1% by brush followed by 300 s of plasma treatment Ar/O_2 0.2% (kINPen).

2.1.4. Characterization. The inoculated samples were observed under a zoom stereo microscope EMZ-8TR (Meiji Techno Co., ltd; ×7–×180 magnification), with both visible and UV light. Digital photos were generated with Nikon D5100 digital camera (AF-S Nikkor 18-55mm) before and after each treatment. The samples were submitted for microbiological analyzes in the Department of Microbiology at Sofia University "St. Kliment Ohridski" and Ca' Foscari University of Venice. After biodeactivation treatments, again in aseptic media, the treated glasses were used for imprints on sterile culture medium (YGC) plates. The plates were cultured at 26°C to determine the presence/absence of growth.

2.2. Consolidation

2.2.1. Samples. Two types of samples were considered: (i) samples of stone and (ii) samples of stone covered with a layer of bat guano, simulating the presence of the prehistoric drawings. Both types of samples were prepared in the form of slabs ($7\times7\times3$ cm^3) and cubes (5 cm side). A limestone with colour and mineralogical composition close to that of the cave was selected (Vratsa limestone, Oreshets quarry, Municipality of Vidin, Bulgaria). The samples simulating the drawings were prepared by depositing a layer of sterilized bat guano mixed with a suitable amount of water (guano:water ratio of 1:2 by weight) over the surface of the stone samples. To avoid detachment of the bat guano layer, treatment with the consolidants was carried out 30 minutes after guano application (hence before the guano was completely dried).

2.2.2. Treatments. For the ethyl silicate (ES) treatment, the commercial product "Bio Estel New" by CTS was used. According to the technical data sheet, the product is enhanced with protective ability against microorganisms. The consolidant was applied by 7 brush strokes over a single face of the samples (the face covered with guano, when present) and then left to cure in laboratory conditions (T = 21 ± 2 °C, RH = 50±5%) for 4 weeks. The ammonium phosphate (DAP) treatment consisted in the application of a hydro-alcoholic solution containing 0.1 M DAP + 0.1 mM $CaCl_2$ in 30 vol% ethanol [11] (all chemicals were purchased from Sigma-Aldrich). The solution was applied by 10 brush strokes over a single face of the samples (the face covered with guano, when present), then the samples were wrapped in a plastic film for 48 hours. The samples were then unwrapped, rinsed with water (except for the samples covered with guano) and left to dry in laboratory conditions until constant weight. To investigate whether pre-treatment by plasma may improve consolidation, by increasing the penetration depth thanks to an increase in stone hydrophilicity, part of the sample was subjected to treatment by non-thermal plasma right before consolidant application. For this purpose a dual frequency atmospheric plasma jet device always with a coaxial DBD design - Stylus Plasma Noble (Nadir Srl, Italy) [12], has been used by applying the following parameters: 5W power at 16 KHz, 30W power at 27MHz, Argon gas flow as working gas at 10 L/min, and compressed air as cooling gas at 12 L/min. The stone samples, of 7×7 cm surface, have been manually treated by the operator for a total exposure time of 2 min, by maintaining a constant working distance of 3 mm between the plasma source and the surface.

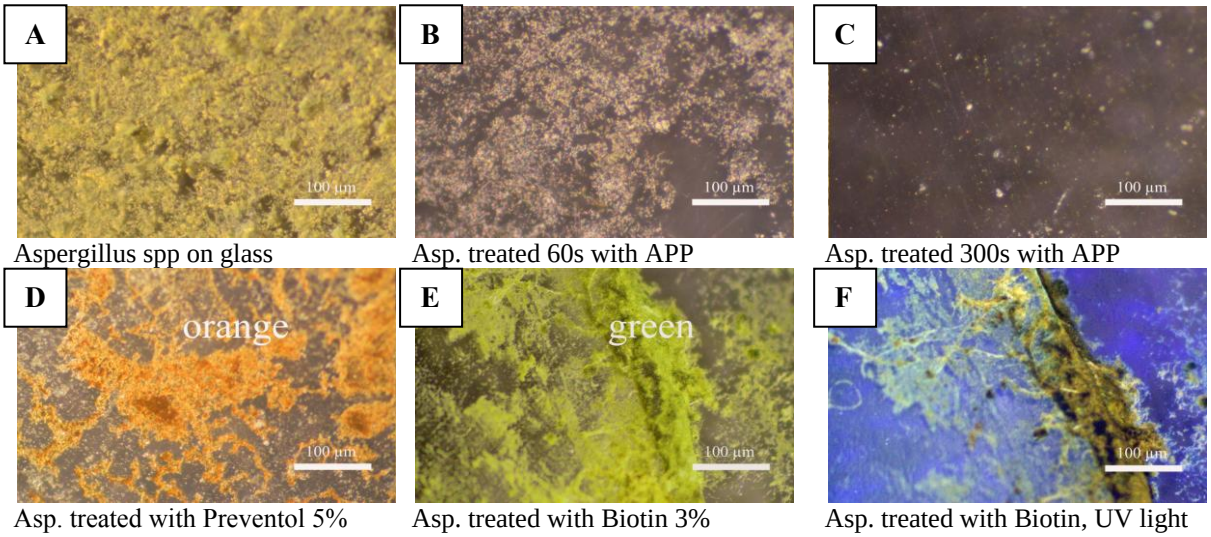
2.2.3. Characterization. The consolidating ability was assessed by first measuring the penetration depth of the two consolidants into the two types of samples, by fracturing freshly treated samples by chisel. The increase in stone cohesion was evaluated by determining the dynamic elastic modulus of the samples, before and after consolidation. The modulus was calculated as the product of the stone density times the ultrasonic pulse velocity to the second power. The ultrasonic pulse velocity was measured by transmission method in the direction perpendicular to the treated face, using a Matest

instrument with 55 kHz transducers and using a rubber to improve the contact between the transducersand the samples. The aesthetic compatibility of the treatments were evaluated by determining the colour change $\Delta E^* = (\Delta L^{*2}+\Delta a^{*2}+\Delta b^{*2})^{1/2}$,where the L*, a* and b* are the LabCIE* colour parameters (L* = black÷white, a* = green÷red, b* = blue÷yellow), determinedusing a NH310 colorimeter. The alteration in water transport properties was assessed by measuring the water absorption by capillarity after 24 h and 7 d, according to the European Standard EN 15801[13] (water being let penetrate the samples through the treated face). The possible benefit deriving from pre-treatment by plasma was evaluated by comparing the contact angle, the penetration depth and the increase in dynamic elastic modulus of samples subjected to consolidation with and without pre-treatment by plasma.

3. Results and discussion

3.1. Biodeactivation

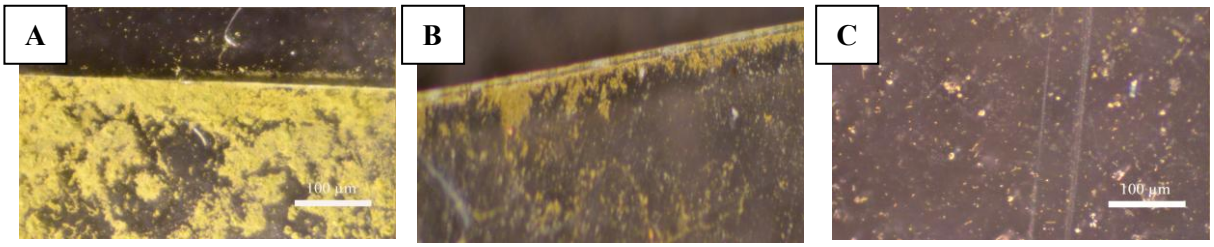
Figure 2 depicts a comparison of non-treated biofilm and biofilm exposed to biocides and Ar/O_2 gas discharge plasma (kINpen). Distinct biofilm removal could be observed only with Ar/O_2 plasma. As can be seen in Fig. 2C, longer plasma exposure time resulted in substantial etching of the biofilm. All control samples exposed to non-ionized Ar/O_2 gas for 60 s and 300 s showed no biofilm etching. Hence, the process gas alone did not result in biofilm removal. The treatment with the two biocides reveals a different activity. Almost no visual removal was obtained after application of Biotin T (3%) and Preventol RI 80 (5%), moreover both biocides alter the colour of the treated samples (Fig. 2D, E, F). The combined method (Preventol RI 80 1%+ kINPen Ar/O_2 (99.8/0.2 %)) demonstrate very satisfying results on the inoculated glass samples. Reduced concentration of Preventol 1% did not alter the colour of the treated surface as Preventol 5% and in combination with plasma sterilization showed efficient deactivation (Figure 3). Microbiological analyses clearly show absence of activity only after treatment with Ar/O_2 plasma and Preventol RI 80 1%+ APP Ar/O_2 (Table 1). Therefore, these two methods were tested on samples representative for the real materials in the Gallery with the paintings (limestone and guano on limestone, inoculated with Penicillium spp./Aspergillus spp., Figure 4). Optical microscopy in combination with the performed microbiological analysis confirm again the successful elimination of micro-organisms by both the treatments. Similarly, APP treatments performed with Stylus Plasma Noble confirmed the biodeactivation of Penicillum spp./Aspergillus spp. after 10 s/cm^2 of plasma treatment performed on glass and petri dishes with grown microorganisms (Table 1).



Aspergillus spp on glass | Asp. treated 60s with APP | Asp. treated 300s with APP

Asp. treated with Preventol 5% | Asp. treated with Biotin 3% | Asp. treated with Biotin, UV light

Figure 2: Biodeactivation treatments on inoculated glass samples 2.5×



Aspergillus spp. on glass | Asp. treated with Preventol 1% | Aspergillus treated with Preventol 1%+APP

Figure 3: Combined method of biodeactivation (Preventol RI 80 1%+ kINPen Ar/O_2 (99.8/0.2 %)) 2.5×

Stone+guano sample, inoculated with Penicillium spp. | Penicillium spp. on stone+guano sample; 4.5× (before treatment) | Penicillium on guano, treated 300s with APP, 4.5×

Figure 4: Biodeactivation treatments on inoculated stone+guano samples

Table 1. Microbiological analyzes determinants presence/absence of growth: - absence; + low presence;+++ high presence; / not performed

Biodeactivation methods	presence/absence of growth							
	Glass+ Penic.	Glass+ Asp.	Stone+ Penic	Stone+ Asp.	Stone+ guano+ Penic.	Stone+ guano+ Asp.	Petri dishes +Penicillum	Petri dishes +Aspergillum
Biotin 3%	+++	+++	/	/	/	/	/	/
Preventol 5%	+	+	/	/	/	/	/	/
kINpen 0.2 %	-	-	-	-	-	-	/	/
Preventoll%+ kINpen0.2 %	-	-	-	-	-	-	/	/
Nadir 0.1%	-	-	/	/	/	/	-	-

3.2. Consolidation

The results of the tests aimed at evaluating the effectiveness and the compatibility of the two consolidating treatments are summarized in Table 2. In the stone samples, the DAP treatment was able to penetrate more deeply than the ES product (7.0 and 4.5 mm, respectively). In the samples covered with guano, the guano layer slightly reduced the penetration depth of the ES treatment (from 4.5 to 3.5 mm), likely because it acted as a barrier. In the case of the DAP treatment, the penetration depth could not be visually assessed, because no wet fringe was clearly visible. In terms of mechanical consolidation, in all cases the two treatments were able to increase the dynamic elastic modulus of the samples, but the improvement was higher in the case of DAP. Compared to the stone samples, in the samples covered with guano the dynamic modulus always resulted lower (also in the case of the untreated references), likely because the guano layer worsened the contact between the ultrasonic transducers and the samples. In any case, mechanical improvement was achieved without any significant colour change of the stone samples ($\Delta E^* = 2.1\text{-}2.7$). In the case of the samples covered with guano, higher values of colour change were registered ($\Delta E^* = 3.3\text{-}5.1$) and, most of all, cracking of the guano layer and its partial detachment were observed in some areas. A major difference

between the two consolidants was the effect onto the water transport properties. While the DAP treatment caused only minor alterations of the water absorption at all times, because very little amounts of new calcium phosphates were formed inside the pores, the ES treatment left the stone hydrophobic even 4 weeks after its application. Consequently, the water absorption after 24 h was dramatically reduced, which can be an issue in practical situations if a water source is present behind the consolidated layer. After prolonged contact with water (7 d), the hydrophobicity of the ES-treated samples is however lost. With regard to the effect of plasma pre-treatment, a significant decrease was registered in the static contact angle measured on the stone surface, which reached almost to zero after plasma treatment. Nonetheless, the treatment penetration depth and the increase in dynamic elastic modulus were not significantly improved by the plasma treatment. This is thought to be a consequence of the fact that the wettability of the stone surface was significantly modified, but, inside the pores, the contact angle between liquids and the pore walls was likely unaffected. Consequently, pre-treatment by plasma seems like a promising strategy for conservation treatments that mostly affect the stone surface (e.g., protective treatments), whereas its efficacy as a preliminary step before treatments aimed at penetrating in depth (e.g., consolidating treatments) seems lower.

Table 2 Consolidating ability and compatibility of the two consolidants (ES and DAP) applied onto stone samples and stone samples covered with guano

	Stone			Stone covered with guano		
	Untreated	ES-treated	DAP-treated	Untreated	ES-treated	DAP-treated
Penetrationdepth (mm)	-	4.5±1.0	7.0±1.0	-	3.5±1.0	notclear
Dynamic elastic modulus (GPa)	45.8±2.3	49.6±2.7	51.1±4.5	41.3±3.9	41.8 ± 3.3	44.1 ± 4.5
Colour change(-)	-	2.69	2.11	-	5.13	3.29
Water absorption after 24 h (wt%)	4.9 ± 0.4	1.9 ± 0.8	4.5 ± 0.2	4.4 ± 0.0	0.2 ± 0.0	4.2 ± 0.1
Water absorption after 7 d (wt%)	5.2 ± 0.4	4.6 ± 0.1	4.9 ± 0.3	4.7 ± 0.0	4.1 ± 0.1	4.8 ± 0.0

4. Conclusions

The study is a first step of overall program aiming to propose decision-making tools for the conservation of the Magura paintings. The purpose of tested materials and methods is to deactivate the microbiological action and consolidate the weakened stone without affecting the original surface (stone and guano). For historical monuments and works of art it is also important that the biocide/consolidant do not alter the color or visual aspects. Therefore, the performed laboratory approach permit to assess from the beginning any possible detrimental effect of used materials, and to only further apply those without any negative impacts.

Summarizing, the results of this study provide evidence that non-thermal APP is efficient in deactivating Penicillium spp./Aspergillus spp. biofilms. Owing to its low gas temperature (near or around room temperature) and non-contact treatment, plasma offers the possibility to eliminate micro-organisms on heat and touch sensitive materials (e.g. prehistoric paintings). In addition, it was shown that an almost complete elimination of biofilm was achieved by combination of very low concentration biocide (Preventol RI 80 1%) and plasma sterilization. Microorganisms are capable of rapidly acquiring chemical resistance, therefore the establishment of two successful methods for biodeactivation and prevention is an outstanding contribution.

As for mechanical consolidation, the obtained results point out the high potential of the DAP-based treatment (significant improvement in mechanical properties, lack of significant alterations in the aesthetic appearance and water absorption), but also the need to develop more realistic procedures to apply the bat guano over the stone surface with the aim of resembling the presence of the prehistoric

drawings. In fact, the tendency to cracking and detachment of the guano layer in the artificial samples was much higher than in the case of the real drawings in the cave. This is the first experimental data on the effectiveness of plasma and DAP/ES application on rock art and the long term protection and durability of the proposed innovative solutions has to be evaluated in real conditions.

5. Acknowledgements

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