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MICROALGAE AS BIODETERIOGENS OF STONE CULTURAL HERITAGE: QUALITATIVE AND QUANTITATIVE RESEARCH BY NON-CONTACT TECHNIQUES

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ABSTRACT

Biological colonisation of stone is one of the main problems related to monuments and buildings conservation. It is amply recognised that microalgae have the greatest ecological importance as pioneer colonisers of stone materials, conducting to aesthetic, physical and chemical damages. Their deterioration potential is related with their photoautotrophic nature, using the mineral components of stone substrates and sunlight as energy source without any presence of organic matter.

Stone biodeterioration by microalgae has been assessed by several authors. Most of the employed methodologies for microbial identification and monitoring are time-consuming and require extensive sampling. In addition, the scaffolding and sampling procedures required may also transform the researcher in a biodeteriorating agent itself. In this chapter, non-contact techniques for colonisation detection and monitoring are proposed in order to fulfil the mission of

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heritage preservation. *In vivo* chlorophyll *a* fluorescence and digital image analysis were applied to estimate microalgal biomass and to quantify coverage of limestone samples artificially colonised by algal communities. The results showed that *Ançã* and especially *Lecce* limestones were extensively colonised on their surfaces revealing significant epilithic growth, whereas *Escúzar* and *San Cristobal* limestones were endolithically colonised by photoautotrophic microorganisms.

The easily handled, portable and non-destructive techniques proposed allow the understanding of stone biodeterioration processes avoiding contact and damaging of the objects, which ensures a wide field of application on cultural heritage studies and the design of appropriate conservation and maintenance strategies.

1. INTRODUCTION

Stone materials have been used since the beginning of mankind. Their selection for construction purposes has been driven by questions of durability, availability, workability, cost and appearance. Despite the bewildering variety of stone types, carbonate rocks have been preferentially used as construction material. Among them, limestones were prised for their attractive appearance, ease of quarrying, workability and exceedingly distribution across the Earth's surface. Thus, some of the most remarkable architectural heritage all over the world was built in limestone. Unfortunately, we are confronted with some problems concerning their preservation. Physical and chemical weathering is observed, which induce stone disaggregation and decomposition resulting from material loss (Smith 2003). Several researchers have studied the deterioration processes occurring on limestone surfaces (Maurício et al. 2005; Dionísio 2007; Figueiredo et al. 2007). In many compact limestones, the rate of deterioration may be gradual and, given climatic conditions, largely predictable. However, there are many commonly limestone types which do not decay gradually, but instead experience episodic and sometimes catastrophic breakdown. The problem of understanding the deterioration of limestones is compounded by the large range of intrinsic properties of limestones, and by their varying responses under different climatic and environmental conditions. The interactions between these numerous and synergistically acting factors lead to a dynamic and complex process of physical, chemical and biological deterioration. This last is the cause of many types of deterioration on limestones, through a process referred as biodeterioration. Biological colonisation of cultural heritage assets, especially those exposed to outdoor environment is one of the main problems that curators have to slow down as it constitutes an important risk factor for their conservation. As photoautotrophic organisms, microalgae play an important ecological role integrating the basement of the food chain. Depending on light, carbon dioxide and a few other elements, they are the pioneer colonisers of stone surfaces, forming phototrophic biofilms which can be described as surface attached microbial communities with a clearly present photosynthetic component (Roeselers et al. 2008). Their presence attracts heterotrophic organisms contributing to the development of complex and stratified biofilms, mainly composed of a multilayer of cells embedded in a hydrated

extracellular polymeric matrix which hold the cells together (Morton et al. 1998; Warscheid 2000; Roldan et al. 2003). The vital activities of microalgae, as well as those of the other components of the biofilms, have a great biodeteriorating potential which ranges from purely aesthetic to physical and chemical changes and can lead to the total disaggregation and soiling of the surface, a problem especially important in stone cultural heritage elements such as historic buildings and monuments. When microalgae colonise stone materials, they adopt different survival strategies that usually imply the fast development of variously-coloured surface patinas (Ortega-Calvo et al. 1995). Most of these coloured patinas are produced by microbial organic pigments firmly bound to the stone particles (Urzì and Realini 1998; Alakomi et al. 2004; Gorbushina 2007). The consequence is the formation of greenish to blackish biofilm generated patinas, particularly evidenced on light colour limestones (Krumbein 2004). The colour of the biofilm constitutes generally an important aesthetic damage, but this is not the only adverse consequence of microalgae colonisation on cultural assets. As an example, characteristic patterns like crack formation, micropitting and biogenic mineral deposition were detected by Sarró et al. (2006) due to the development of microalgae on the Lions Fountain at the Alhambra Palace (Granada, Spain).

If the environmental conditions are not the most suitable for life, the strategy of microalgae also imply an endolithic growth on the stone substrate, being this euendolithic when they actively dissolve the stone, cryptoendolithic, when the cells find their niche inside the rock pores and structural cavities, or chasmoendolithic, when the cells find protection on fissures and cracks of the rock (Golubic et al. 1981). The development of endolithic biofilms can produce the detachment of stone surface areas, due to the mechanical action of wetting/drying cycles of the extracellular polymeric substances, or due to chemical action of excreted metabolic products on the substrate (Miller et al. 2010a).

The universally recognised value of cultural assets, whether being stone monuments, archaeological remains, paintings, or others works of art, limits the availability of samples for the scientific study of biodeterioration processes. The need for non-contact techniques is of great importance as the researcher can be transformed itself in a biodeteriorating agent since most methodologies employed for microbial identification and monitoring requires extensive sampling.

In the last years, a number of non-destructive characterisation techniques have been developed in order to be applied on cultural heritage assets. The *in situ* application of analytical techniques previously confined to laboratory, is rather demanded, allowing non-destructive studies without sampling procedures.

In general, algal biomass dwelling on stone monuments can be quantified through chlorophyll *a* quantification techniques. However, most of these methods are based on the extraction of chlorophyll from disintegrated cells in an organic solvent and on its subsequent determination by spectrophotometry (Parsons and Strickland, 1963), fluorometry (Yentsch and Menzel, 1963) and high performance liquid chromatography (Goeyens et al., 1982). In fact, these methods are widely used in limnological researches to analyse phytoplankton, periphyton, marine or freshwater algae, as well as in monitoring programs with the purpose of ecosystem management (Macedo et al., 2000, 2001). Nevertheless, they are time-consuming,

require large volume of samples to follow the temporal dynamics of photosynthetic communities and do not allow the repeated measurement in time of the same sampling unit, because of their destructive nature.

In recent years, a rapid, reliable and non-destructive chlorophyll determination method based on *in vivo* chlorophyll fluorescence was introduced in the analysis of monuments and historic buildings (Cecchi et al. 2000; Tomaselli et al. 2002; Miller et al. 2006). This method is based on the quantification of chlorophyll *a* on solid substrates through the detection of its natural fluorescence, without sampling procedures. Thus, *in vivo* chlorophyll fluorescence has been used to detect phototrophic microorganisms on monuments and to monitor preventive treatments (Cecchi et al. 2000; Tomaselli et al. 2002; Miller et al. 2006).

The quantification and monitoring of algal biofilms on surfaces can also be performed by digital image analysis techniques, which comprise the set of mathematical operations applied to detect, monitor and quantify different elements included in digital images. As digital image, we understand every pictorial representation of the data obtained by a sensor, i.e., a device capable for detecting electromagnetic radiation, for converting it into a signal and for presenting it in a picture (Chuvieco Salinero 2002). The data obtained by a sensor is directly related to the materials reflectance, which is the percent of reflected radiation in its sensitivity wavelength range. These data are translated to numerical values and ordered in a matrix in which two Cartesian coordinates define the spatial position and a third coordinate defines the reflectance value. Digital images are usually multiband images, as for the Cartesian coordinates, is commonly available more than one reflectance coordinate. This set of two Cartesian coordinates and a third reflectance coordinate receives the name of band. A typical digital photographic image is composed of three bands, each one with encoded reflectance values of the intervals 400-500, 500-600, and 600-700 nm (Blue, Green and Red bands). Thus, digital image analysis is the set of mathematical operations that can be performed with this type of images. These techniques have been used to improve the visualisation of rock art motifs (Rogerio-Candelera 2008), and to record separately different elements (in terms of nature and composition) present in mural paintings (Rogerio-Candelera et al. 2011).

In this chapter, digital image analysis was applied in combination with *in vivo* chlorophyll *a* fluorescence as rapid, reliable and non-contact techniques for the detection and monitoring of biodeterioration processes on different limestone types. With both techniques, qualitative but also quantitative information of microalgal biomass was obtained with the advantages of being low time-consuming and without the need of contact or sampling.

2. Materials and Methods

2.1. Colonisation Experiment

The usefulness of quantifying algal biomass by non-contact techniques was illustrated by a laboratory-based stone colonisation experiment, in which five limestone types were inoculated with a culture of microalgae and cyanobacteria and incubated in a climatic chamber (Miller et al. 2010b). The limestone types tested were:

Ançã limestone (CA) – a Portuguese fine-grained, compact to oolitic tendency limestone from the Jurassic;

Lioz limestone (CL) – a Portuguese microcrystalline, very fine-grained limestone from the middle Cretaceous with stylolite joints and recrystallised bioclasts;

San Cristobal stone (SC) – a Spanish coarse-grained calcarenite of the Upper Miocene;

Escúzar stone (PF) – a Spanish heterogeneous and coarse-grained biocalcarenite from the Tortonian;

Lecce stone (PL) – an Italian fine-grained Miocene limestone, almost exclusively composed by sparite bioclasts and scarce cementation.

Before inoculation, replicates of each limestone type (3 cm height and 4.4 cm diameter) were sterilised at 120ºC and 1 atm for 20 min. After cooling, the upper surface of the stone samples were inoculated with a multiple-species phototrophic culture composed of microalgae and cyanobacteria, described and tested in a previous study (Miller et al. 2009). The inoculated stone samples were immediately placed in a climatic chamber at 20±2ºC and 12h dark/light cycles during 90 days of incubation. Detailed information regarding the laboratory-based colonisation experiment, as well as the petrographic and petrophysical characteristics of each lithotype, is presented in Miller et al. (2010b).

The laboratory-induced colonisation on initially uninhabited limestones presented in this chapter was achieved by inoculating stone samples with a multiple-species community culture since in nature microorganisms involved in stone biodeterioration develop in more or less complex communities because of the diversity of rock ecosystems. Consequently, the choice for the stone inoculation comprised a community of phototrophic microorganisms that are potential deteriorating agents of the selected stone materials. Furthermore, the use of a complex microbial community presents the advantage to simulate the existence of competition and/or synergy between colonising microorganisms, which act singly or in association with other microorganisms, or with physicochemical factors, to deteriorate stones (Koestler et al. 1996). In addition, the stone samples were not re-inoculated and no extra nutrients were added during the experiment. These procedures allowed the comparison of the temporal development of microalgae colonisation on five different limestones throughout two non-destructive photosynthetic biomass quantification techniques.

2.2. Quantification of Photosynthetic Biomass during the Colonisation Experiment

In vivo chlorophyll *a* fluorescence and digital image analysis were applied and compared in order to quantify and monitor the development of photosynthetic growth

on the stone samples during the incubation time. Chlorophyll *a* is a photosynthetic pigment present in all photoautotrophic microorganisms, including microalgae and cyanobacteria, used to estimate the amount of photosynthetic biomass present in liquid media, in soil and also on rock substrates. Moreover, this pigment is in the origin of green-coloured patinas, which is a good indicator of the presence of algal biofilms. Hence, the temporal dynamic of microalgae colonisations dwelling on stone substrates can be quantified by means of surface areas covered by these green-coloured biofilms using digital image analysis.

In Vivo Chlorophyll a Fluorescence Technique

The growth of phototrophic microorganisms on the stone samples was assessed by *in vivo* chlorophyll *a* fluorescence method. This is a non-destructive, very fast, safe and easy method for the estimation of phototrophic biofilms dwelling on solid substrates, without the extraction of chlorophyll *a* from disintegrated cells. Fluorescence properties of some compounds, such as the natural fluorescence of chlorophyll *a*, are detected with a spectrofluorometer, providing their intensity of fluorescence in counts per second (cps) which give information of their concentration in a sample. A certain excitation wavelength is selected, and a scan is performed to record the intensity versus wavelength, called an emission spectra. Chlorophyll *a* absorbs light in all regions of the visible spectrum, showing maximum absorption in the blue-violet (about 430 nm) and red regions (around 660 nm) and emitting in a wavelength of about 680 nm when light excited at 430 nm.

Stone samples of each lithotype were taken out of the chamber in triplicate at the inoculation time and after each 30 days of incubation (0, 30, 60 and 90 days). Emission spectra were determined using a spectrofluorometer SPEX Fluorolog-3 FL3-22 fitted with a fibre-optic platform (Horiba Jobin Yvon F-3000). For each stone sample five spectrofluorometric measurements were randomly carried out on the surface of the stone samples covered by the biofilm. The fibre-optic end-piece was held steady facing the sample surface at a distance of 2 mm. Measurements were performed with an excitation wavelength of 430 nm (optimum for chlorophyll *a* molecules, APHA/AWWA/WEF, 1992), slits of 4.5 nm, an integration time of 0.3 s and an increment of 1.0 nm.

Digital Image Analysis

Digital image analysis techniques constitute a low-cost and very useful set of tools allowing non-destructive recording and quantification of different elements included in digital images even when they are not recognised by eye. In this chapter, digital image analysis was focused on the detection and quantification of stone surface areas covered by algal biofilms along the incubation time. Three replicates of each lithotype were taken out of the climatic chamber after 0, 45 and 90 days of incubation and placed on millimetric paper under controlled light to ensure fixed conditions for all photographic records. The photographic recording was performed with a digital camera Kodak EasyShare P850. The generated RGB digital images recorded at different incubation times were transferred and processed on a personal computer in order to digitally rectify the geometry of the images, since they should constitute comparable series both geometrically and radiometrically making them

consistent for comparative purposes. Radiometric corrections have been performed adjusting the maximum and minimum pixel values. Geometric corrections are necessary due to the different distortions introduced by several factors as the kind and structure of the employed lenses, the focal distance or the relative position of the photographic camera. In most cases, it is necessary the employ of digital photogrammetry techniques to ensure the geometrical consistence of the series. In this chapter, the comparability of the images has been ensured by means of the same lighting conditions, focal length and normal position of the camera respect to the samples. Adobe Photoshop® software was used for the digital rectification of single photographs as shown by Mark and Billo (1999). The result of these geometric corrections is a multi-layer file, in which each layer corresponds to one of the incubation stage recorded.

After radiometric and geometric rectifications, digital decorrelation of RGB images by means of Principal Components Analysis (PCA) technique and simple image classification by the application of a thresholding algorithm were performed.

Image decorrelation by PCA allows the contrast enhancement of digital images (Gillespie et al. 1986), avoiding the loss of information implicit in the methods which redefine the histograms, as the ones known as *linear*, *histogram*, or *special* stretches (Lillesand and Kiefer 2000), and also eliminating the alteration of pixel values obtained by the application of digital filtering. This make these images as suitable for image classification as the original ones, because the transformation experienced by the pixel values is purely geometric, being these linear combinations of the original values (Chuvieco Salinero 2002). One of the main explanations of the spectral differences detected in the different Principal Components bands even in optically homogenous RGB images is that they reflect different compositions. This assumption leads to the application of PCA to issues as mineral survey by means of satellite imagery (Loughlin 1991), the differentiation of phases in rock art paintings (Rogerio-Candelera et al. 2009), or the improvement in the visualisation of rock art panels, even if some figures are not visible at all (Portillo et al. 2008). The Principal Components of a digital image are calculated by means of the expression:

$$PC_j = \sum_{i=1,p} a_{ij} DN_i + R_j \qquad (1)$$

where PC_j represent the pixel value corresponding to Principal Component j, $a_{i,j}$ is the coefficient applied to the pixel value of the band i in order to generate the component j and R_j a constant introduced in each component in order to avoid negative values.

Therefore, this approach allows the detection of minority elements (of different nature and composition) apparently absent in the initial RGB digital image but masked by the redundant data registered in the Red, Green and Blue bands of the image. With this decorrelation it is possible to choose the most appropriate band corresponding to each PC (PC1, PC2 or PC3) which enhance the visualisation of the photosynthetic biomass present on the stone surfaces. In our case, the colour and texture of some limestones might mask the presence of the algal biofilms on their surfaces, which could not be detected without the digital decorrelation of the images. This approach was performed using the HyperCube v. 9.5 software (*Army Geospatial*

Centre, Alexandria, Virginia, USA). The application of an iterative thresholding algorithm was then considered necessary to segment the images into binary, allowing the selection of the colonised areas to be quantified. The binarisation of the images is based in the recognition of the extreme pixel values of the coverage visually identified as biomass. Typically, iterative thresholding algorithms work using the average of the foreground and background class means, establishing a new threshold (T_n) by iteration (Sezgin and Sankur 2004). The algorithm employed displays the threshold according to:

$$T = (g_{\max} - g_{\min}) \sum_{g=g_{\min}}^{g^*_{mid}} p(g) \qquad (2)$$

where g_{max} is the highest nonzero grey level, g_{min} is the lowest one, g_{mid} is the midpoint between the two assumed points of the histogram [$g_{mid}=(g_{max}+ g_{min})/2$], and p(g) the probability mass function. For area estimation, the images were scaled, and the selected pixels counted. This allowed obtaining a series of numerical values, permitting the estimation of growth rates along the experimental period. All these image operations were performed using the ImageJ v. 1.38x software (*National Institutes of Health*, Bethesda, MD, USA).

3. Results and Discussion

3.1. Photosynthetic Biomass Quantified by *In Vivo* Chlorophyll *a* Fluorescence

For the evaluation of the algal colonisation process during the incubation time span, *in vivo* chlorophyll *a* fluorescence was measured on the surface stone samples immediately after inoculation and after 30, 60 and 90 days of incubation. The initial fluorescence intensities obtained for CA and PL abruptly increased during the first 30 days of incubation (Figure 1a). The *in vivo* chlorophyll *a* fluorescence values after 30 days-incubation for SC and PF samples were also about three times higher than those immediately after inoculation. In general, the chlorophyll *a* fluorescence intensities increased during the first 30 days of incubation for all lithotypes, and decreased after 60 days of experimentation, with the exception of SC lithotype (Figure 1a).

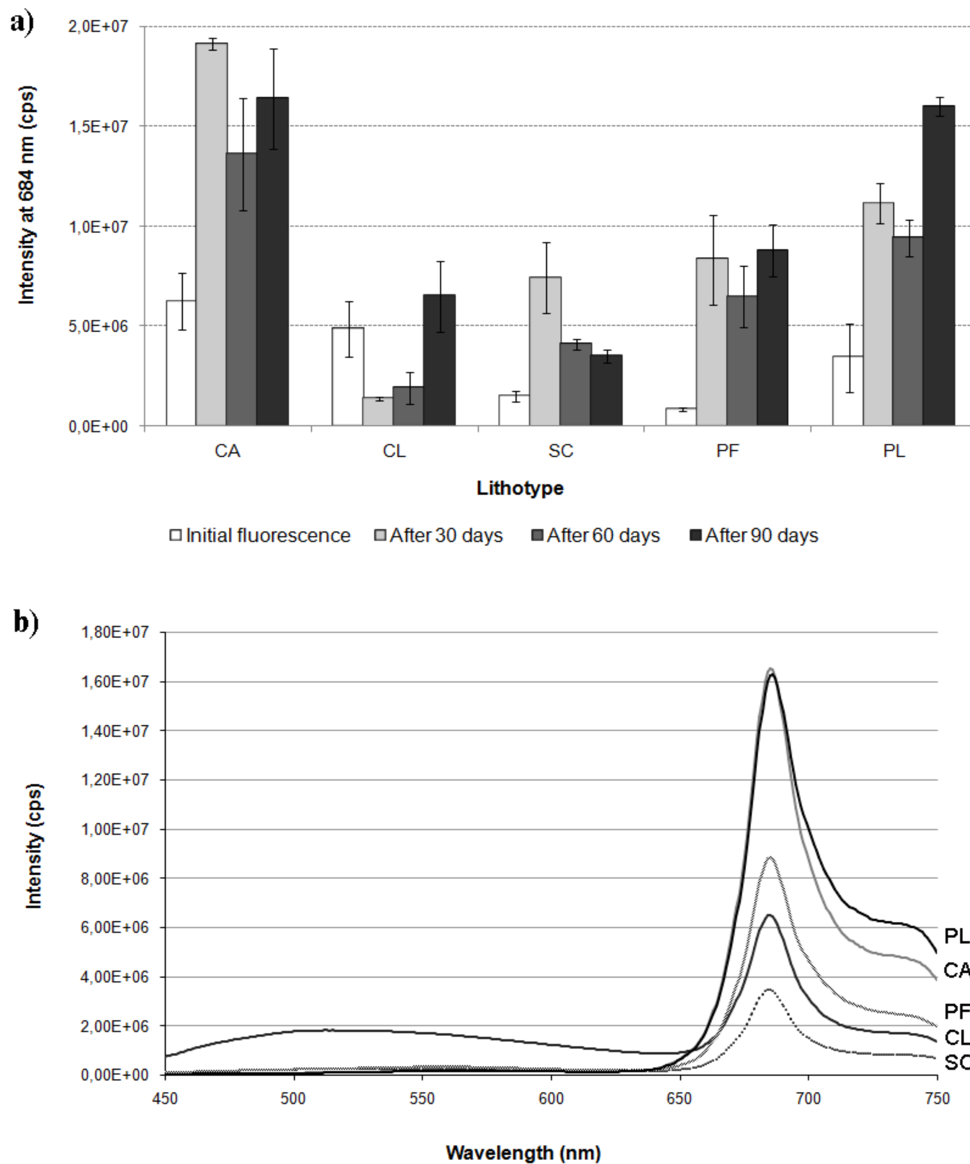

Figure 1. Intensities of chlorophyll *a* fluorescence obtained for each lithotype (excitation wavelength: 430 nm): A) Fluorescence intensity values of chlorophyll *a* at 684 nm measured immediately after inoculation (initial fluorescence), and after 30, 60 and 90 days of incubation. Each column corresponds to the mean value of an average of 15 measurements ± SD. B) Chlorophyll *a* fluorescence spectra measured after 90 days of incubation. Each lithotype spectrum is an average of 15 spectra.

This high development of green biofilms after 30 days of incubation, was conceivably due to residual culture medium (BG11) elements present in the inoculum, providing nutrients for microbial growth. In contrast, on CL surfaces this development was not observed probably due to the very compact nature of this limestone, hindering the inoculum absorption into the samples. In fact, it was verified

that after 60 days-incubation a decrease occurred for all lithotypes surfaces except for CL (Figure 1a). This could be attributed to the lack of nutrients provided by total consumption of the elements present in the inoculum and to a negative adaptation to the new type of nutrients supplied by the lithic substrates. However, if growth were only determined by the culture medium elements, similar results would be obtained in all lithotypes and no re-increase of microbial biomass would occur as observed for CA, PF and PL limestones. On the other hand, for CL a great decrease was observed until 60 days of incubation, after which an increase was observed until the end of the incubation experiment. Indeed, CL was the limestone depicting the lowest quantity of chlorophyll *a* during the firsts 60 days-incubation, which tended to increase during the last 30 days of incubation. This suggests that the phototrophic colonisation would progressively increase if the incubation period were extended. According to Roeselers et al. (2006), the end of exponential growth does not necessarily mean that a stable climax community has established or cessed. The biofilm may be still in an adaptation state, developing slowly towards a final convergence.

The intensity of chlorophyll *a* fluorescence recorded for the medium-grained SC lithotype decreased after 60 days of incubation until the end of the experiment (Figure 1a), being the least colonised lithotype after 90 days-incubation. This significant decrease noticed to SC samples indicated apparent cessation of epilithic colonisation.

After the inoculum development observed during the first 30 days, PF was the only lithotype where the mean values of chlorophyll *a* fluorescence remained approximately the same during the 90 days of experimentation. This result suggests an adaptation state of the microalgae colonisation to this stone substrate.

The emission spectra obtained after 90 days for all limestone types showed the typical chlorophyll *a* fluorescence peak at 684 nm (Figure 1b). After 90 days, high fluorescence intensities were obtained for CA and PL lithotypes which presented visible biofilms formed on their surfaces. High fluorescence intensities represent a high quantity of photosynthetic biomass on the surface of the stone samples. These results obtained for CA and PL lithotypes were probably due to their fine grained textures and petrophysical characteristics (Miller et al. 2010b). As verified in Figure 1b, SC samples showed the lowest quantity of chlorophyll *a* and thus the lowest algal development on their upper surfaces.

3.2. Stone Coverage Areas Quantified by Digital Image Analysis

For the evaluation of the colonisation process during the incubation time span, the measurement of areas covered by the algal biofilms by means of digital image analysis was also performed. This approach allowed the monitoring of biofilm development on stone samples of the five limestone types throughout the quantification of stone surface areas covered by the green biofilms. By means of the thresholding algorithms applied to bands obtained by PCA (Figure 2), it was possible

to isolate the areas covered by the biofilm and quantify the phototrophic cover through the time as represented in Figure 3.

Figure 2. Thresholded areas obtained by ImageJ software for CA: A) After inoculation; B) After 45 days incubation; C) After 90 days incubation. The detected particles are then measured using ImageJ software.

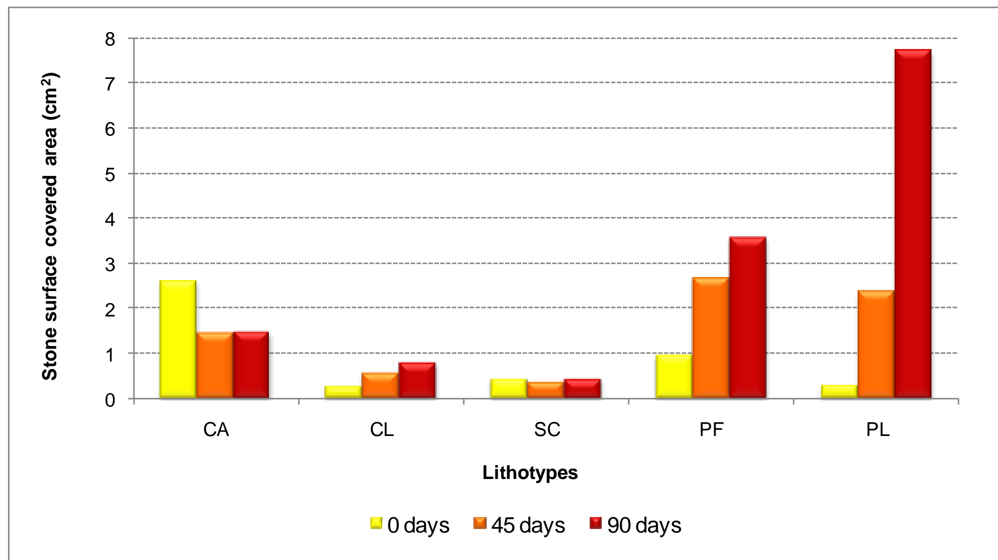

Figure 3. Stone surface areas covered by algal biofilms after 0, 45 and 90 days of incubation, quantified by digital image analysis.

According to this complementary visual monitoring technique, it was possible to assess which samples showed the most extensively colonised surfaces, i.e., a significant epilithic growth. The phototrophic culture, randomly distributed on the lithic surfaces, has grown during the incubation course leading to an increase of surface covered areas. CL showed a very slight increase of surface area covered by growth, also noticed by the *in vivo* chlorophyll *a* fluorescence technique, suggesting the progressive increase of algal colonisation if the incubation were extended. In spite of the difficulties for measuring phototrophic biofilms on PF and SC sample surfaces, strongly masked by the high macroporosity of these lithotypes, the digital image analysis approach was successful since it allowed the quantification of stone surface

coverage areas. For SC samples, total surface area covered by the phototrophic biofilm did not show an increase over the course of batch incubation, being the least colonised surface samples among the studied lithotypes, as also observed by *in vivo* chlorophyll *a* fluorescence technique. Distinctively, it was noticeable the epilithic growth registered for PF, which showed a significant increase of phototrophic colonisation on its surfaces; the stone surface covered area was greater than after inoculation, showing a progressive increase during the experiment. As in the case of *in vivo* chlorophyll *a* fluorescence, CA showed a great increase of algal biofilm after the inoculation time, decreasing after 60 days of incubation. A re-increase of biomass was not observed by digital image analysis after 90 days-incubation, as noticed by *in vivo* chlorophyll *a* fluorescence.

According to both approaches, PL samples showed extensive colonised surfaces, revealing significant epilithic growth, followed by PF lithotype. In contrast, SC showed the lowest microalgal biomass. Nevertheless, according to the data presented by Miller et al. (2010) in which *in vitro* chlorophyll *a* quantification technique was combined with *in vivo* chlorophyll *a* fluorescence to analyse the five limestone types, SC and PF were the most colonised stone substrates. The authors concluded that endolithic growth occurred for these lithotypes as revealed by optical and electron microscopy of transversally cut stone samples (Miller et al. 2010b). Gathering all these data together it can be corroborated that the non-destructive techniques used in this chapter can only detect and quantify phototrophic biofilms displayed on the stone surfaces and not growing inside them. Hence, the combination of *in vivo* chlorophyll *a* fluorescence and digital image analysis techniques gave a rather good presentation of algal biomass variation and provided qualitative and quantitative evaluations of epilithic phototrophic growth on the limestones studied. Therefore, it can be concluded that on CA, PL and CL lithotypes algal colonisation occurred epilithically, whereas on SC and PF samples, the microbial growth occurred mainly inside the stone samples.

Both techniques, even though they are non-destructive and produce rapid measurements and quantified observation in less time than conventional methods, are nevertheless insufficient to detect and evaluate endolithic growth without destroying the sample.

Conclusion

Experimental simulations investigating stone colonisation are commonly used in ecological studies since they provide a valuable alternative for natural ecological niches by allowing experimental manipulation of the microbial ecosystem. The laboratory-based studies are of great interest for the particular case of cultural heritage materials, as is the case of the study presented in this chapter.

Our results illustrate the suitability of non-destructive methods as digital image analysis and *in vivo* chlorophyll *a* fluorescence to monitor the development of microalgae colonisations on limestone materials, even in an incipient stage when it is difficult to visually appreciate the green colour characteristic of chlorophyll. The most

important advantage of the use of these methods is their non-invasivity, which allows obtaining qualitative and quantitative data of repeated samples along time without sampling procedures, and thus to contribute to the elaboration of adequate conservation strategies for cultural heritage assets. Due to the detection of phototrophic microorganisms at an early stage of development on stone surfaces, the use of *in vivo* chlorophyll *a* fluorescence and digital image analysis is also considered as an important tool to control possible relapse. Probably it would not be hazardous to state that the generalisation of the use of these techniques would be of great interest for researchers, conservators and, in last instance, for cultural assets itself. Nevertheless, endolithic growth is not detected by these techniques, which represents a major obstacle when an integral study of the stone phototrophic colonisation is needed.

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REFERENCES

1. Alakomi, H.L., Arrien, N., Gorbushina, A.A., Krumbein, W.E., Maxwell, I., McCullagh, C., Robertson, P., Ross, N., Saarela, M., Valero, J., Vendrell, M., Young, M.E. 2004. Inhibitors of biofilm damage on mineral materials (Biodam). In: Kwiatkowski, D., Löfvendahl, R. (Eds.) Proceedings of the 10th International Congress on Deterioration and Conservation of Stone, ICOMOS, Stockholm, pp. 399-406.
2. Alcolea, J.J., Balbín, R. 2007. C^{14} et style. La chronologie de l'art pariétal à l'heure actuelle. *L'Anthropologie* 111: 435-466.
3. American Public Health Association (APHA), American Water Works Association (AWWA), Water Environmental Federation (WEF), 1992. Standard methods for the examination of water and wastewater, 18th edition, APHA/AWWA/WEF, Washington, D.C.
4. Cecchi, G., Pantani, L., Raimondi, V., Tomaselli, L., Lamenti, G., Tiano, P., Chiari, R., 2000. Fluorescence lidar technique for the remote sensing of stone monuments. *Journal of Cultural Heritage* 1, 29-36.
5. Chuvieco Salinero, E., 2002. Teledetección ambiental. La observación de la Tierra desde el Espacio. Ariel, Barcelona.
6. Dionísio, A., 2007. Stone decay induced by fire on historical buildings: the case of the Cloister of Lisbon Cathedral (Portugal). In: Prikryl, R., Smith, B.J. (Eds), Building Stone decay: from diagnosis to conservation. *Geological Society*, London, Special Publication 271, 87-98.

7. Figueiredo, C.A.M., Aires-Barros, L., Basto, M.J., Graça, R.C., Maurício, A., 2007. The weathering and weatherability of Basílica da Estrela stones, Lisbon, Portugal. In: Prikryl, R., Smith, B.J. (Eds), Building stone decay: from diagnosis to conservation. *Geological Society*, London, Special Publication 271, 99-107.
8. Gillespie, A.R., Kahle, A.B., Walker, R.E., 1986. Color enhancement of highly correlated images. I. Decorrelation and HSI contrast stretches. *Remote Sensing of Environment* 20, 209-235.
9. Goeyens, L., Post, E., Dehairs, F., Vandenhoudt, A., Baeyens, W., 1982. The Use of High Pressure Liquid Chromatography with Fluorimetric Detection for Chlorophyll A Determination in Natural Extracts of Chloropigments and their Degradation Products. *International Journal of Environmental Analytical Chemistry* 12, 51-63.
10. Golubic, S., Friedmann, I., Schneider, J., 1981. The lithobiontic ecological niche, with special reference to microorganisms. *Journal of Sedimentary Petrology* 51, 475-478.
11. Gorbushina, A.A., 2007. Life on the rocks. *Environmental Microbiology* 9, 1613-1631.
12. Krumbein, W.E., 2004. Life on and in stone – an endless story? In: Kwiatkowski, D., Löfvendahl, R. (Eds), Proceedings of the 10th International congress on deterioration and conservation of stone. ICOMOS, Stockholm, pp. 259-266.
13. Lillesand, T.M., Kiefer, R.W., 2000. Remote Sensing and Image Interpretation. Fourth Edition. John Wiley and sons, New York.
14. Loughlin, W.P., 1991. Principal component analysis for alteration mapping. *Photogrammetric Engineering and Remote Sensing 57,* 1163-1169.
15. Macedo, M.F., Duarte, P., Alves, M., Ferreira, J.G., Costa, V., 2000. Analysis of the deep chlorophyll maximum across the Azores Front. *Hydrobiologia* 441, 155-172.
16. Macedo, M.F., Duarte, P., Mendes, P., Ferreira, J.G., 2001. Annual variation of environmental variables, phytoplankton species composition and photosynthetic parameters in a Coastal Lagoon. *Journal of Plankton Research* 23, 719-732.
17. Maurício, A., Pacheco, A., Brito, P., Castro, B., Figueiredo, C., Aires-Barros, L., 2005. An ionic conductivity-based methodology for monitoring salt systems in monument stones. *Journal of Cultural Heritage* 6, 287-293.
18. Miller, A., Dionísio, A., Macedo, M.F., 2006. Primary bioreceptivity: A comparative study of different Portuguese lithotypes. *International Biodeterioration and Biodegradation* 57, 136-142.
19. Miller, A.Z., Laiz, L., Dionísio, A., Macedo, M.F., Saiz-Jimenez, C. 2009. Growth of phototrophic biofilms from limestone monuments under laboratory conditions. International *Biodeterioration and Biodegradation* 63, 860-867.
20. Miller, A.Z., Rogerio-Candelera, M.A., Laiz, L., Wierzchos, J., Ascaso, C., Sequeira Braga, M.A., Hernández-Mariné, M., Maurício, A., Dionísio, A., Macedo, M.F., Saiz-Jimenez, C. 2010a. Laboratory-induced endolithic growth

in calcarenites: biodeteriorating potential assessment. *Microbial Ecology* 60, 55-68.
21. Miller, A.Z., Leal, N., Laiz, L., Rogerio-Candelera, M.A., Silva, R.J.C., Dionísio, A., Macedo, M.F., Saiz-Jimenez, C. 2010b. Primary bioreceptivity of limestones applied on Mediterranean Basin monuments. In: B.J. Smith, M. Gomez-Heras, H.A. Viles, J. Cassar (Eds.) Limestone in the Built Environment: Present Day Challenges for the Preservation of the Past. Geological Society, London, Special Publications 331, 79-92.
22. Morton, L.H.G., Greenway, D.L.A., Gaylarde, C.C., Surman, S.B., 1998. Consideration of some implications of the resistance of biofilms to biocides. *International Biodeterioration and Biodegradation* 41, 247-259.
23. Ortega-Calvo, J.J., Ariño, X., Hernandez-Marine, M., Saiz-Jimenez, C., 1995. Factors affecting the weathering and colonization of monuments by phototrophic microorganisms. *Science of the Total Environment* 167, 329-341.
24. Parsons, T.R., Strickland, J.D.H., 1963. Discussion of spectrophotometric determination of marine pigments with revised equations for ascertaining chlorophylls and carotenoids. *Journal of Marine Research* 21, 155-63.
25. Portillo, M.C., Rogerio Candelera, M.A., Gonzalez, J.M., Saiz-Jimenez, C. 2008. Estudios preliminares de la diversidad microbiana y análisis de imagen de las manifestaciones parietales en los abrigos de Fuente del Trucho y de Muriecho L (Colungo, Huesca). In: S. Rovira, M. Garcia-Heras, M. Gener, I. Montero (Eds.) Actas del VII Congreso Ibérico de Arqueometría. CSIC, Madrid, pp. 97-107.
26. Roeselers, G., van Loosdrecht, M.C.M., Muyzer, G. 2008. Phototrophic biofilms and their potential applications. *Journal of Applied Phycology* 20, 227-235.
27. Rogerio-Candelera, M.A., 2008. Una propuesta no invasiva para la documentación integral del arte rupestre. M Sc Thesis, University of Seville.
28. Rogerio-Candelera, M.A., Vanhaecke, F., Resano, M., Marzo, P., Porca, E., Alloza Izquierdo, R., Sáiz-Jiménez, C., 2009. Combinación de análisis de imagen y técnicas analíticas para la distinción de diferentes fases en un panel rupestre (La Coquinera II, Obón, Teruel). In: J.A. López Mira, R. Martínez Valle, C. Matamoros de Villa (Eds.) El Arte Rupestre del Arco Mediterráneo de la Península Ibérica. 10 años en la lista del Patrimonio Mundial de la Unesco. Generalitat Valenciana, Valencia, pp. 327-334.
29. Rogerio-Candelera, M.A., Jurado, V., Laiz, L., Saiz-Jimenez, C., 2011. Laboratory and in situ assays of digital image analysis based protocols for biodeteriorated rock and mural paintings recording. *Journal of Archaeological Science* DOI:10.1016/j.jas.2011.04.020.
30. Roldan, M., Clavero, E., Hernández-Marine, M., 2003. Aerophytic biofilms in dim habitats. In: Saiz-Jiménez, C. (Ed), Molecular biology and cultural heritage. A.A. Balkema, Lisse, pp. 163-169.
31. Saiz-Jimenez, C., Ariño, X. 1995. “Biological colonization and deterioration of mortars by phototrophic organisms”. *Materiales de Construcción* 45(240): 5-16.

32. Sarró, M.I., Garcia, A.M., Rivalta, V.M., Moreno, D.A., Arroyo, I., 2006. Biodeterioration of the Lions Fountain at the Alhambra Palace, Granada (Spain). *Building and Environment* 41, 1811-1820.
33. Sezgin, M., Sankur, B., 2004. Survey over image thresholding techniques and quantitative performance evaluation. *Journal of Electronic Imaging* 13, 146-165.
34. Smith, B.J., 2003. Background controls on urban stone decay: lessons from natural rock weathering. In: Bimblecombe, P. (Ed), The effects of air pollution on the built environment, *Air pollution reviews, Vol. 2*, Imperial College Press, pp.31-61.
35. Tomaselli, L., Lamenti, G., Tiano, P., 2002. Chlorophyll fluorescence for evaluating biocide treatments against phototrophic biodeteriogens. *Annals of Microbiology* 52, 197-206.
36. Urzì, C., Realini, M., 1998. Colour changes of Noto's calcareous sandstone as related to its colonisation by microorganisms. *International Biodeterioration and Biodegradation* 42, 45-54.
37. Warscheid, T., 2000. Integrated concepts for the protection of cultural artifacts against biodeterioration. In: Ciferri, O., Tiano, P., Mastromei, G. (Eds) Of microbes and Art – The role of microbial communities in the degradation and protection of cultural heritage, Kluwer Academic, New York, pp. 185-201.
38. Yentsch, C.S., Menzel, D.W., 1963. A method for determination of chlorophyll and phaeophytin by fluorescence. *Deep-Sea Research* 10, 221-31.