

A first insight on the biodegradation of limestone: the case of the World Heritage Convent of Christ

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Abstract The present study is a multidisciplinary approach applied to architectural stone materials of the Convent of Christ in Tomar (Portugal) in order to understand and mitigate the active decay processes. The structure and appearance of the stonework from the Convent of Christ are strongly affected by stains, biofilms and structural degradation. To investigate these phenomena, a multianalytical approach comprising X-ray microdiffraction, scanning electron microscopy, microRaman and microinfrared spectroscopy was applied to the examination of altered outdoor stone areas being detected calcium oxalates, carotenoids and microbial proliferation. The presence of these alteration products seems to be correlated with the microbial activity of bacteria, microalgae, cyanobacteria and filamentous fungi. This work showed that the application of complementary methodologies is an efficient strategy to characterise the stone decay, and constitute a starting point for successful conservation intervention plans that are urgent to ensure

the preservation and safeguard of this emblematic monument.

1 Introduction

The usage of stone in artworks goes back to our ancestors due to the inherent characteristics of this material, particularly their high resistance, durability and versatility. There is a wide diversity of stones like limestone, granite, marble, basalt and sandstone that are used in built heritage and monuments. However, carbonate stones (marble and limestone) are the most common components of many objects of importance for cultural heritage like statues, tombstones, historic buildings and archaeological sites due to their intrinsic characteristics [1, 2].

Despite their resistance, stone materials are subjected to unfavourable natural and anthropogenic factors that modify the appearance of the surfaces and their integrity. Thus, stone buildings decay can be promoted by intrinsic factors like composition, structure, texture, porosity and particle size, and/or extrinsic factors such as environmental conditions and location of the building [3, 4].

The rock surfaces located in outdoor environments are exposed to weathering processes, which is the result of the action of chemical, physical and biological agents, that promote formation of secondary minerals and the production of biominerals and/or erosion mechanisms [3, 5, 6], inducing loss of stone material, discoloration/deposits, detachment and fissures/deformation.

In the last years, only decay processes associated with freeze–thaw and soluble salts crystallisation cycles as well as chemical attack by acid rain and wind erosion were taken into account in the stone materials deterioration, being the biological agents action almost neglected [3].

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However, recent studies revealed the contribution of microorganisms—algae, cyanobacteria, lichens, fungi and bacteria—as potentially harmful agents for stone artworks [7, 8]. Cyanobacteria and algae are pioneer organisms, which colonise habitats potentially unavailable for most living organisms and transform them, allowing the colonisation of other groups of microorganisms. Their development adversely alters the aesthetic aspect and structural integrity, affecting the historic and cultural value of the monuments [9, 10]. In this way, the investigation of the microbial population dynamics present in rock artworks and the interpretation of their role in the biodeterioration/biodegradation is crucial for the safeguard of Cultural Heritage.

The Convent of Christ in Tomar, Portugal (twelfth century to sixteenth century), classified as UNESCO World Heritage, is an emblematic monument with a wide diversity of sculpted stone, particularly in the Cloisters and in the Manueline window, which possess visible alteration signals that, in a near future, may affect the integrity of this monument. Several architectural styles like Romanesque, Gothic, Manueline, Renaissance, Mannerist and Baroque can be found in this cultural, touristic and devotional space, whose preservation may be threatened [11]. The stone materials present in this Convent show evident structural and aesthetic damages like biofilms formation, coloured stains, delamination and detachment of stone fragments (Fig. 1) which need to be understood. According to this, the main goal of this work was to perform a comprehensive multidisciplinary approach to understand the reasons for the alteration and degradation of the Convent of Christ stone materials.

2 Materials and methods

2.1 Sampling process

The sampling process was performed on representative areas of the stone materials present in the Convent of Christ, and also with significant contamination and alteration signs, under the supervision of a conservator-restorer.

Microsamples of stone materials (ten samples with $<1\text{ mm}^3$) for material characterisation were removed using a small chisel, near losses or cracks to avoid further damage. For microbiological assays, samples were collected under semi-aseptic conditions with sterile swabs and scalpels and placed in a suspension of transport MRD medium (Maximum Recovery Diluent, Merck), until utilisation.

2.2 Material characterisation and products alteration detection

2.2.1 X-ray diffraction

X-ray microdiffraction (μ -XRD) analyses were performed on a Bruker AXS D8 Advance with a DAVINCI design diffractometer, equipped with a Göbel mirror assembly and a LynxEye 1D detector. Cu $K\alpha$ radiation and 0.3 mm diameter pinhole collimator were used for this study. The angular range (2θ) was scanned from 3° to 75° at a step size of $0.02^\circ/s$, with a working voltage and current of 40 kV and 40 mA, respectively. The XRD patterns deconvolution and matching were performed with Bruker EVA software using the International Centre for



Fig. 1 Stone materials of Convent of Christ with evident alteration signs like coloured stains (a, b), structural degradation with fragments detachment (b) and biofilms formation (c) selected to evaluate their decay process

Diffraction Data Powder Diffraction Files (ICDD PDF). The semi-quantification of the identified crystalline phases was performed by a Rietveld quantification routine using the same software.

2.2.2 *MicroRaman spectroscopy*

Raman spectra were acquired in a HORIBA Xplora Raman microscope, coupled to external power laser sources for specimen radiation: 638 nm (He–Ne) and 785 nm (diode laser). Samples irradiation was performed using a filter 10–50% to prevent any thermal damage of the material. Ordinary acquisition time was of the order of 10–20 s with 5 cm^{-1} of spectral resolution. The backscattered light is collected by the objective (10× or 50×), and then captured by a CCD (Charge Coupled Device) detector. Spectra were calibrated using the 520.5 cm^{-1} band of a silicon wafer. Spectra deconvolution was performed using LabSpec and the identification was made with Spectral IDTM 13.

2.2.3 *Fourier transform infrared spectroscopy with the attenuated total reflection*

Infrared spectra were obtained on a Bruker ALPHA FTIR instrument, equipped with the attenuated total reflection (ATR—QuickSnap) set-up coupled with crystal diamond were used to complement the characterisation of the materials. To obtain a good signal-to-noise ratio, 128 scans were accumulated for each spectrum at a spectral resolution of 4 cm^{-1} , between 4000 and 375 cm^{-1} . Spectral analysis was performed with OPUS 6.0 software.

2.2.4 *Scanning electron microscopy coupled with energy-dispersive x-ray spectrometry (SEM–EDS)*

To assess the micromorphology and composition of the stone materials, the deterioration degree of the support and the signalisation of microbial contamination, microsamples of stone were used as such or coated with Au–Pd (Balzers Union SCD 030) during 30 s, and observed in an HITACHI S-3700N variable pressure scanning electron microscope (VP-SEM) with accelerating voltage of 10–20 kV. Microanalyses of the selected samples were performed using the same microscope coupled with a Bruker XFlash 5010 energy-dispersive X-ray spectrometer (EDS) to allow microstructural characterisation of the mortars and elemental composition (point analysis and 2D mapping). EDS analyses were performed at 20 kV.

2.3 Microbial communities

The samples collected for biological assays were mechanically shaken during 24 h at 120 rpm. After this

period, each sample was inoculated in solid cultures containing different culture media, selective for several kinds of microorganisms. Bacterial isolation procedures were carried out in Nutrient Agar, at $30\text{ }^{\circ}\text{C}$, for 48 h. The distinct single colonies obtained were subcultured onto NA Petri dish and maintained on NA slants at $4\text{ }^{\circ}\text{C}$. For fungal colonies isolation, two standard mycological media (Malt Extract Agar—MEA and Cook Rose Bengal—CRB) were used and the cultures incubated for 7 days at $28\text{ }^{\circ}\text{C}$.

The different microbiological strains were identified following standard methods [12], based on their macro- and micromorphological characteristics.

3 Results and discussion

Several built stone areas of the Convent of Christ reveal different structural and aesthetic damages that strongly affect the aspect and the integrity of the structures which can lead serious problems on the stone materials, and be dramatic for the monument safeguard. For this reason, it is important to explore the factors that induce the alterations on this rock surfaces. Thus, the study performed in the stone materials of Convent of Christ affected by biofilms formation, coloured stains and structural degradation (Fig. 1) involved a pluridisciplinary and multianalytical approach in order to characterise the materials composition and alteration products formed, under the actual environmental conditions.

3.1 Chemical analysis

XRD is a convenient and useful tool for the identification of crystalline compounds allowing the assessment of the mineralogical composition of stone substract, salts and alteration products and has been applied in the field of built heritage conservation.

XRD analyses of the stone materials showed typical patterns of limestone with calcite as main crystalline phase and quartz (Fig. 2). Particularly, relevant was the identification of alteration products in areas with visible biological colonisation including calcium oxalates, whewellite ($\text{CaC}_2\text{O}_4\cdot\text{H}_2\text{O}$) and weddellite ($\text{CaC}_2\text{O}_4\cdot 2\text{H}_2\text{O}$) and gypsum (Fig. 2; Table 1). Calcium oxalates are the result of the reaction between oxalic acid exudate by microorganisms and calcium present on the stone either as a constituent element (calcite) or as accidental deposit (calcium salts) [13, 14]. Gypsum, on the other hand is the result of the reaction of stone calcite with the combustion and exhaust gases (SO_2).

The widespread presence of oxalates on stone materials in several climatic conditions has led to much discussion about their formation processes, having two main

Fig. 2 XRD diffractogram of stone microfragments from Convent of Christ with evident alteration signs (*c* calcite, *g* gypsum, *q* quartz, *wh* whewellite, *w* weddellite)

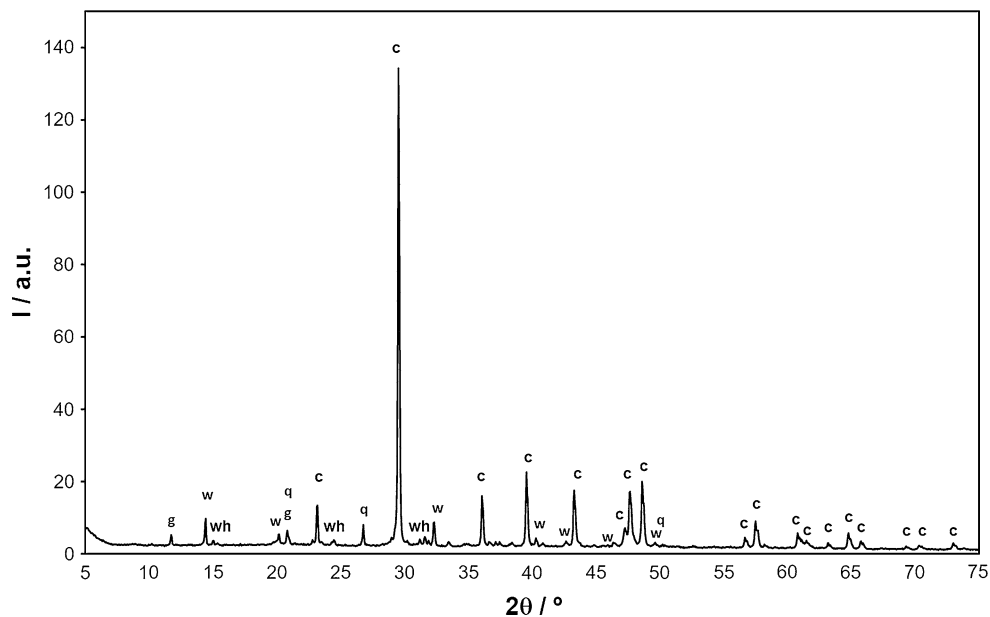


Table 1 Summary of X-ray diffraction results performed on stone microfragments from altered areas with coloured stains (a, b), structural degradation with fragments detachment (b) and biofilms formation (c)

Area	Compound name	Relative percentage (%)
<i>a</i>	Calcite	89
	Quartz	2
	Weddellite	9
<i>b</i>	Calcite	81
	Gypsum	3
	Quartz	4
	Weddellite	10
	Whewellite	2
<i>c</i>	Calcite	89
	Quartz	11

hypotheses for their origin—biological and chemical. According to recent investigations [14, 15], the biological origin of calcium oxalates is associated with the occurrence of microorganisms like fungi and bacteria on the stone surfaces, which produce oxalic acid as metabolic product that react with calcium present on the stone. Calcium oxalates formation is very stable, less soluble, and hardly affected by atmospheric pollution, influencing the integrity of the artwork [13].

The presence of calcium oxalates and other alteration products on biofilm zones was also detected by Raman analysis (Fig. 3b). This spectroscopic tool allows also to identify carotenoids on stain areas (Fig. 3a) by the spectral profile constituted by bands at 1000, 1100 and 1500 cm^{-1} [16–18]. The production and excretion of carotene

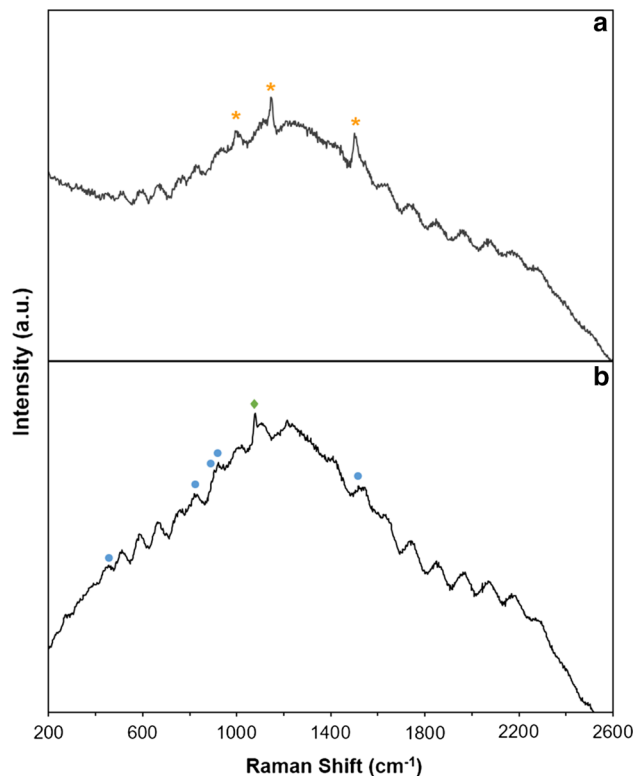
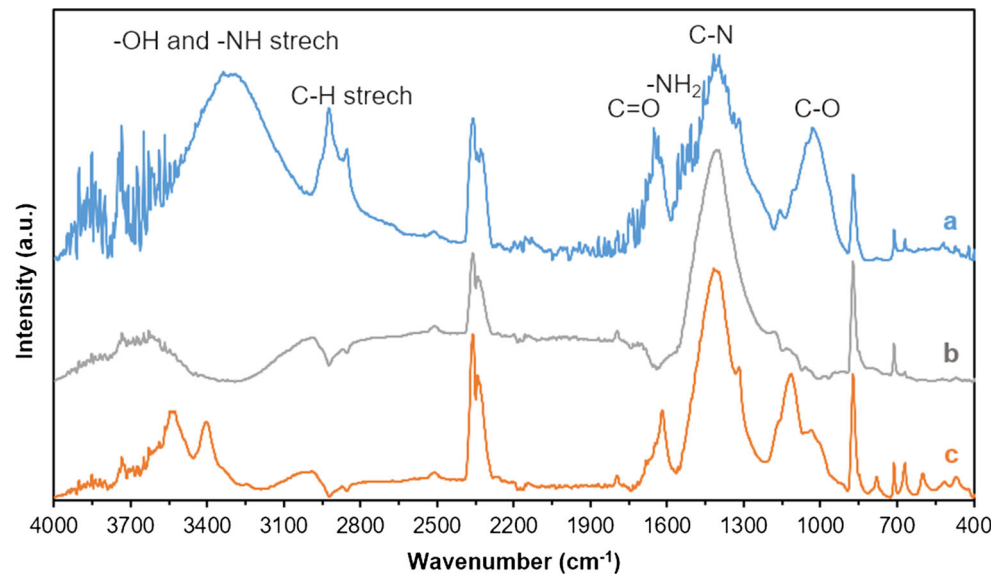


Fig. 3 Raman spectra evidencing carotenoids (a) and calcium oxalates (b) presence on altered rock areas of the Convent of Christ (orange color asterisk carotenoid compounds, blue color filled circle calcium oxalate compounds, green color filled diamond calcite)

compounds can be an indication of microbial colonisation of the stone materials, which needs to be explored.

To complement the results obtained, FTIR–ATR analysis were also performed, allowing the detection of

Fig. 4 FTIR–ATR analysis of several microfragments collected from coloured stains (a), structural degradation with fragments detachment (b) and biofilms formation (c) of Convent of Christ



biomolecular peaks as it follows through the tentative assignments (Fig. 4): broad and strong band situated in the range $3000\text{--}3500\text{ cm}^{-1}$ can be attributed to overlapping of --OH and --NH stretching (3350 cm^{-1}); band around

2920 cm^{-1} attributed to C--H stretching vibrations and around $2280\text{--}2390\text{ cm}^{-1}$ characteristic of microorganisms signature, together with a band near 900 cm^{-1} ; and C=O stretching in carboxyl or amide groups at

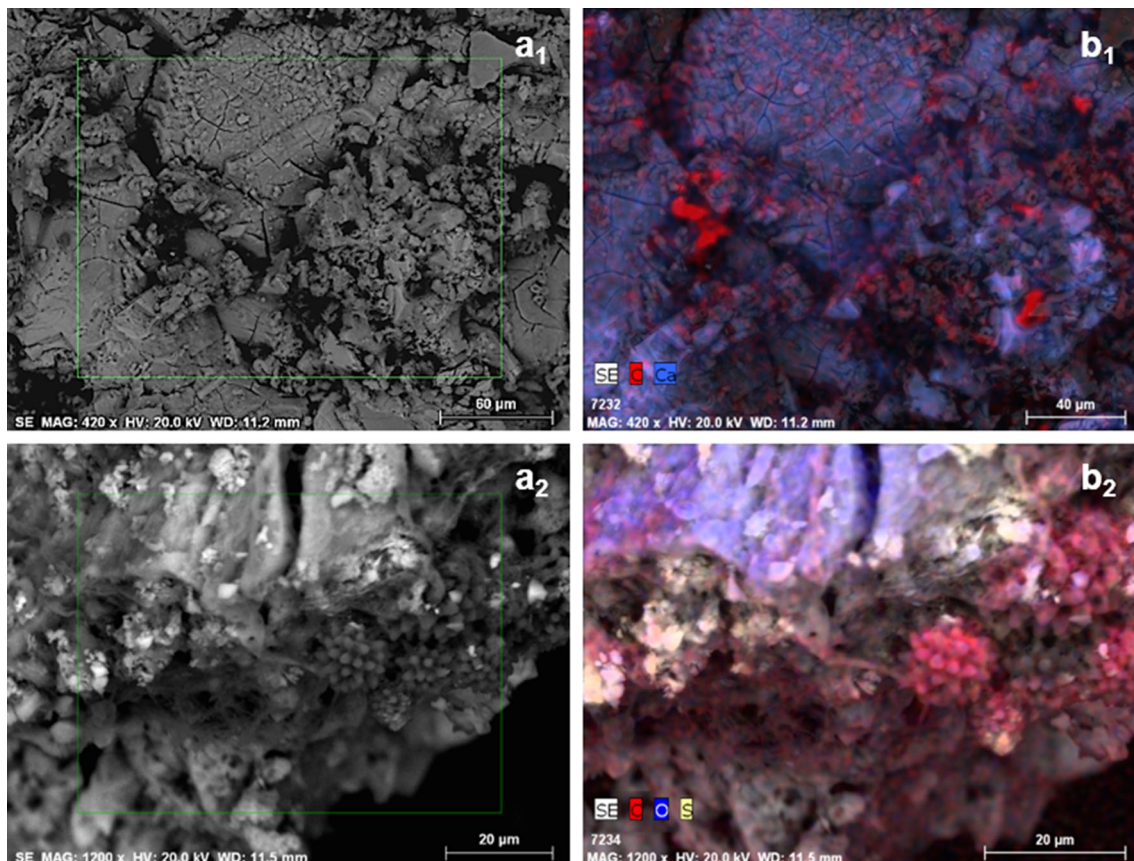


Fig. 5 Backscattered image of stone microfragments (**a**₁, **a**₂) from Convent of Christ obtained by SEM–EDS analysis with 2D distribution maps (**b**₁, **b**₂)

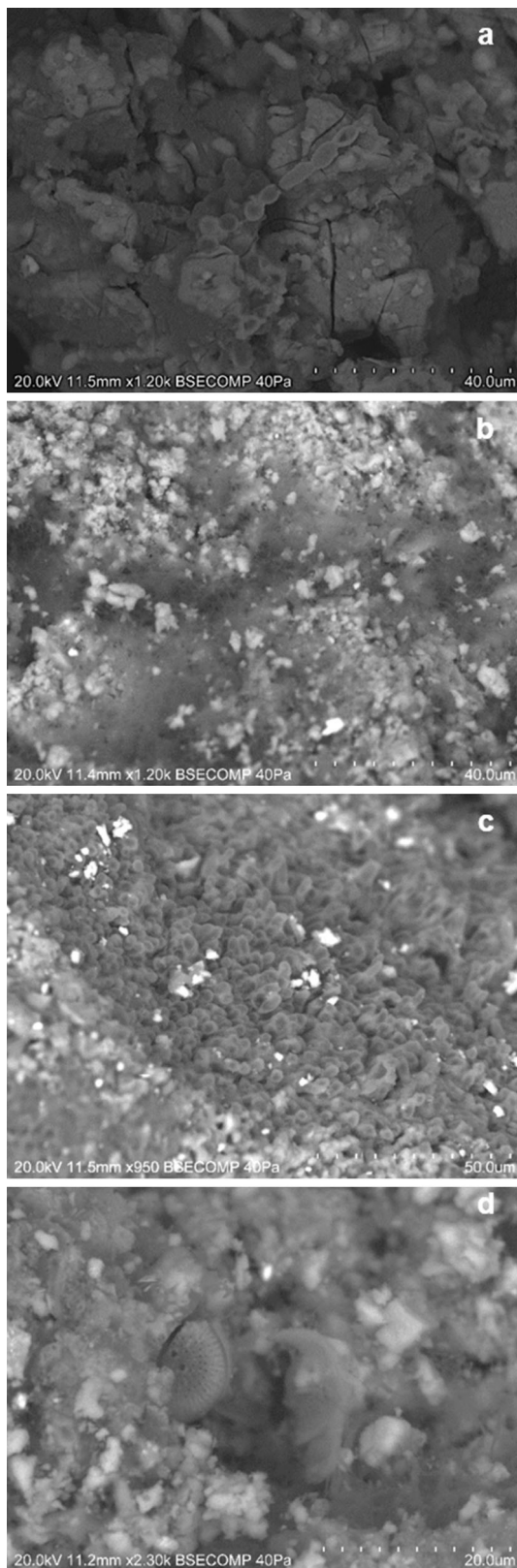


Fig. 6 Scanning electron micrographs of microbial population that thrive on stone altered areas of the convent (**a** bacteria, **b** filamentous fungi biofilm, **c** cyanobacteria, **d** microalgae)

1615–1640 cm^{-1} . The peak at 1404–1405 cm^{-1} is attributed to N–H bending in amine group, and the band observed at 1073–1077 cm^{-1} was assigned to the CO stretching of alcohols and carboxylic acids, which can be associated with the presence of organic material.

In this way, the alteration products detected by XRD and Raman spectroscopy, seems to be correlated with rock biocolonisation, confirmed by IR biological fingerprint (Fig. 4). The results showed the stretching and bending vibrations of molecular bonds or functional groups present in its proteins, nucleic acids, lipids, sugars, and lipopolysaccharides, corroborating the presence of contamination on the stone materials and their active role on the decay of these materials.

3.2 Biocolonisation assessment

EDS analysis performed in the stone microfragments (Fig. 5) shows the presence of organic material on the rock surface, due the combination of elemental components like carbon, sulphur and oxygen (Fig. 5b₂), confirming the spectral evidences of organic compounds obtained by FTIR and Raman analysis. These micrographs allow also to discriminate the stone material from the organic material, through the signalisation of calcium on the rock surface (Fig. 5b₁).

This result was further corroborated by micromorphological analyses (Fig. 6), where it is clear the biological contamination, being possible to observe the proliferation of bacterial cells (Fig. 6a), fungi (Fig. 6b) and cyanobacteria and/or microalgae (Fig. 6c, d). The scanning electron microscopy technique is a powerful magnification tool providing topographical, morphological and compositional information giving three-dimensional images with high resolution. The wide diversity of microorganisms detected seems to be responsible for: (1) the coloured stains due to the deposition of metabolic products on the rock surfaces, and (2) the formation of biofilms, mainly constituted by filamentous fungi, which have the capacity to obtain nutrients from the rock, affecting their cohesion and inducing some cracks and even detachment of some fragments.

This microscopic analysis is indispensable for cultural heritage studies, giving information about the materials composition, deterioration assessment, microbial proliferation, types of colonising agents and preservation/alteration status of the artwork.

Culture-dependent methods were also applied to complement the indications obtained by SEM analysis, which enabled to characterise the cultivable population that inhabit the rock materials of the Convent of Christ. The predominant cultivable microbial contaminants identified were bacteria, cyanobacteria, yeast and filamentous fungi

of the genera *Penicillium* and *Cladosporium*. To overcome some drawbacks of the culture-based methods, due this approach detect <1% of the microbial communities present in the Earth [19], complementary DNA-based approaches are in progress, to complement the information until now and to obtain a more complete information about the biocolonisers present in the Convent, which will be decisive for future rehabilitation studies.

On the other hand, the incidence of biological contaminants in the most deteriorated areas needs to be exploited and integrated with biochemical and biogeophysical studies in order to clarify the role of the microorganisms in the decay process.

Furthermore, it is necessary to be mindful with the biological communities' dynamics, changes in species composition and colonisers succession on medium to long timescales, in order to outline an effective conservation plan and long-term solutions to the biodeterioration of cultural resources.

4 Conclusions

The application of complementary analytical methodologies—XRD, μ -Raman, μ -FTIR, SEM and SEM-EDS—are valuable tools for characterisation, surfaces analysis, assessment of biocolonisation and weathering effects on stone materials of cultural heritage monuments.

Alteration products like calcium oxalates and carotenoids were identified on deteriorated areas of the stone materials present on Convent of Christ, which seems to be correlated with the presence of colonisation as the result of their metabolic activity.

Fungi of the genus *Penicillium* are the main cultivable microorganisms detected on the areas with biofilms formation, while in the stain areas, the prevalent biocolonisers are bacteria and cyanobacteria, whose fully identification is in progress.

The techniques used are very useful and have a powerful impact due to the capacity for covering the biotic and abiotic aspects being an added-value approach to characterise the materials and to diagnose/identify the problem that induce decay processes.

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