Journal of Cultural Heritage 36 (2019) 275–285



Review

# Limestone biodeterioration: A review on the Portuguese cultural heritage scenario

Ana Catarina. Pinheiro [a,*], Nuno Mesquita [a], João Trovão [a], Fabiana Soares [a], Igor Tiago [a], Catarina Coelho [a], Hugo Paiva de Carvalho [a], Francisco Gil [b,c], Lidia Catarino [d], Guadalupe Piñar [e], António Portugal [a]

[a] Centre for Functional Ecology, University of Coimbra, Coimbra, Portugal
[b] Center for Physics of the University of Coimbra (CFisUC), Coimbra, Portugal
[c] UI&D Molecular Chemical-Physics UC 'QFM-UC, Coimbra, Portugal
[d] Geosciences Center, University of Coimbra, Coimbra, Portugal
[e] University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

ARTICLE INFO

*Article history:*
Received 8 May 2018
Accepted 11 July 2018
Available online 23 August 2018

*Keywords:*
Limestone
Biodeterioration
Microorganisms
Portugal

ABSTRACT

Stone, one of the earliest testimonies of human artistic expression, is susceptible to biodeterioration by microorganisms. The most frequent stone colonizing agents are algae, cyanobacteria, bacteria, fungi and lichens, each with their own set of adaptive traits, which allow them to prosper and consequently damage the stone substrate. Limestone is particularly susceptible to biological agents; therefore, in order to act towards the protection and prevention of colonization by microorganisms, it is crucial to understand the microbial communities thriving in limestone heritage buildings. Data regarding the biodiversity and biological activity in Portuguese limestone monuments is, however, still scarce and the scattered knowledge on the subject impairs a full comprehension of the complex and relevant phenomena associated with this particular setting. This review presents and discusses the available studies performed in Portuguese limestone. In addition, the state of the art methodologies to be used, as well as the future studies to be considered, in order to effectively protect such invaluable witnesses of our history, are discussed.


## 1. Introduction

From pre-historic caves to modern buildings, stone has sturdily served our protection needs and our aesthetic cravings. Stone located outdoors is especially susceptible to biodeterioration processes promoted by the colonization of microorganisms related to the different types of stone (such as limestone, sandstone and marble) and the inherent stone bioreceptivity (i.e. the proneness to colonization by living organisms) [1] either on the surface (epilithic colonization), the inner cavities (endolithic colonization), or both [2]. The most frequent stone colonizers are algae, cyanobacteria, bacteria, fungi and lichens all able to damage the stone substrate by physical, aesthetic or other alterations. Their footprint is especially relevant when stone structures are part of our cultural heritage, thus requiring expensive and urgent preventive and conservative measures [3].

Several reviews have been elaborated on the relationship between microorganisms and stone monuments [2,4–10] most of them at an international level, but none focusing specifically on Portuguese monuments, and the characteristic limestones used in their construction. In addition, very few articles discuss the inherent stone characteristics that define their bioreceptivity, as these relate not only to the stone structure [11] but also to its petrophysical characteristics [12], chemical composition, pH [1,13] and state of conservation. Since weather and climatic changes have synergistic effects with air pollution, these should also be considered when discussing bioreceptivity [5,6,14,15].

Carbonate rocks, such as limestones, are prone to biodeterioration [12,16], revealing a high intrinsic bioreceptivity. Natural weathering in temperate climates is responsible for 1.5-3 mm of limestone surface erosion within a 100 years period [17], but when considering all the potentially involved microorganisms (free-living bacteria, archaea, fungi, algae and the symbiotic lichens), a more severe damage is expected. The mechanisms of microbial deterioration can vary from the formation of biofilms to discoloration, salting, physical damage as well as production of osmolytes and organic acids [18].

* Corresponding author.
*E-mail address:* catmarpin@gmail.com (A.Catarina. Pinheiro).

https://doi.org/10.1016/j.culher.2018.07.008
1296-2074/

This chapter gathers available information on Portuguese limestone monuments, their biodeterioration agents, effects and studied features that allow biodeterioration to occur. Although data are presented for just one country the effects are extrapolatable to other countries of the Mediterranean basin. Methods for the assessment of established biological communities are also briefly discussed.

## 2. Limestones and their main features

Limestone is a sedimentary rock composed mostly of calcite and aragonite, both comprised of calcium carbonate ($CaCO_3$), but assembled in a trigonal and orthorhombic system, respectively [19]. Limestone can also contain quartz, sand, chert and clay. The presence of the mineral dolomite in higher proportions is responsible for the designation of a particular type of limestone: dolomitic limestone or dolomite.

Some of the features that turn carbonate rocks (limestones in particular) into good targets for biodeterioration are the same features that make them an historic favourite for the construction and ornamentation of our Cultural Heritage – a soft, bright and easy to carve rock [20]. There are several regional areas in Portugal where limestone is abundant, but according to the region of rock extraction different colour assortments can be obtained.

The Portunhos limestone (from which Ançã is a subtype) is a homogenous and pure limestone with relative weight proportion of $CaCO_3$ superior to 96.5 percent. Its colours range from white to grey, and usually display an oolitic tendency. Due to its low compressive strength and hardness, it was extensively used in Portuguese monuments [12]. Also, the fact that it is bright, very homogenous and finely grained, makes it very suited for highly detailed carvings [20].

Dolomitic limestone (also termed dolostone) is composed of both $CaCO_3$ and the mineral dolomite, a calcium magnesium carbonate [$CaMg(CO_3)_2$] mineral. According to the proportion of magnesium and iron contents, the displayed colours can range from greyish-light brown to a yellowish tone [21,22]. A yellow toned dolomitic stone (low magnesium and high iron content) has extensively been used in the region of Coimbra [21,23] (Fig. 1).

The Lioz limestone is usually extracted from the northern region of Lisbon, and is made of pure calcite that displays a coarser grain and a colour palette that varies between whitish-yellow and bluish-grey [22]. Fig. 2 displays three examples of monuments where the mentioned limestones were used.

**Fig. 1.** Two types of limestone (yellow and white) were used for the construction and punctual rehabilitation of the Old Cathedral in Coimbra.

Physical and chemical features of limestones have been thoroughly studied [15,21,22,24,25]. Some of these features are determinant to the behaviour of this stone in harsh climates (frost-defrost cycles), since crevices may be formed on their surface, providing ideal niches for microorganisms to flourish. The Lioz limestone presents the best characteristics to withstand such conditions (measured in terms of resistance to compression and/or flexion and water absorption rate) followed by dolomite and Portunhos limestone [22]. The bioreceptivity of stone is also influenced by factors such as pH, porosity, texture, roughness, moisture, density and the chemical composition of the surface layer [1,15]. Therefore, the determination of such physicochemical parameters provides researchers with a relevant insight into the susceptibility of the support to microbial attack.

Miller et al. [16] compared two limestone types in terms of their primary bioreceptivity, and described a higher susceptibility of the Portunhos – Ançã limestone when compared with the Lioz limestone, which is in agreement with previous data reported by Carvalho et al. [22] concerning porosity. Although dolomite was not considered in the referred study, its chemical and physical profile should place it between the two types studied by Miller et al. [16] regarding primary bioreceptivity.

Interpreting the available data on the characteristics of limestone could serve as a tool for the prediction of biological colonization; nonetheless this is still limited by the lack of available studies focused on Portuguese limestones.

## 3. Biological colonization of limestone

A wide variety of organisms and microorganisms can be found in different limestones. They can be organized according to their nutritional group or taxonomic classification. The separation into phototrophic, chemoorganotrophic and chemolithotrophic relates to the way these organisms can obtain energy. Algae and cyanobacteria belong to the phototrophic group since they can derive their energy from the sunlight. Chemolithotrophs are microorganisms able to use inorganic substrates (e.g., ammonia, nitrites, hydrogen sulphide, thiosulfates or elementary sulphur) to obtain energy, which results in the release of nitrous acids (e.g., *Nitrosomonas* spp.), nitric acid (nitrifying bacteria, e.g., *Nitrobacter* spp.) or sulfuric acid (sulphur-reducing bacteria) [5,18]. Fungi, actinomycetes and non-filamentous bacteria break chemical compounds for energy purposes but use only organic substrates to do so – they belong to the chemoorganotrophic group. For all the organisms mentioned above, obtaining their carbon from the atmosphere makes them autotrophs, while heterotrophs use organic sources for carbon.

The available studies on limestone display a wide information about consistent populations comprising some of these groups (frequently fungi, algae and lichens), but other groups of organisms, such as bacteria and archaea, are completely overlooked. In fact, to date, there is no published information regarding archaeal diversity on Portuguese limestones. *Archaea* have gone unnoticed due to their difficulty to be isolated as pure cultures [26] and because molecular biology protocols optimized for studying *Bacteria* have been the popular choice in most molecular studies [26–28]. In an effort to bypass this issue recent advances in molecular protocols have enabled to turn the spotlight on *Archaea* and their possible effects on stone biodeterioration [18,26,28–30]. In this domain, there are potentially relevant taxa belonging to the halophilic and alkaliphilic groups [2], which might find their perfect habitat in stones, where salt efflorescence is common.

*Bacteria* constitute a large domain of prokaryotic microorganisms and are known to discolour, disrupt, weaken and dissolve a wide variety of materials [6]. Broadly speaking, their acidic metabolic by-products induce corrosion, and the monitoring of

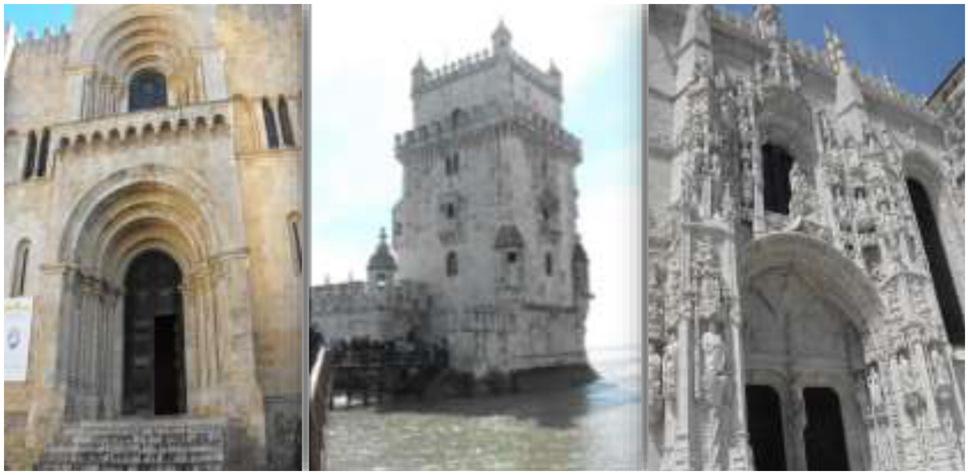

**Fig. 2.** In the Coimbra area, Ançã limestone and dolomitic limestone were frequently used. The Old Cathedral (left) displays both types. In the Lisbon area, where the Belém Tower (center) and the Jerónimos Monastery (right) are located, the Lioz limestone was used instead.

the resulting decrease in pH has already been appointed as a tool to predict and prevent biodeterioration [13]. On the other hand, this domain can also display a positive contribution, since bacteria such as *Bacillus cereus* and *Mixococcus xanthus* can induce calcium carbonate precipitation and therefore act as consolidants in these supports [31–34]. *Bacteria* can display different nutritional pathways; the most commonly associated with deterioration processes are mainly sulphur-reducing bacteria and actinomycetes, and both have been identified in indoor artworks, and less involved in the deterioration of outdoor monuments [35]. However, some species belonging to the order *Actinomycetales* have also been isolated from monumental stones [18,35].

Different nutritional pathways can be found in the *Proteobacteria* Phylum. While *Alphaproteobacteria* are generally phototrophic, *Beta* and *Deltaproteobacteria* are either nitrifying or sulphur and iron reducing bacteria (chemolithotrophic).

It is known that chemical weathering accelerates colonization of sandstone by nitrifying bacteria, and that they are dependent on water availability [5] being abundant in indoor environments and during rainy seasons [18]. Endolithic nitrifying bacteria were the first microorganisms proposed as the cause of stone decay, but because most natural stones are alkaline in reaction, nitrification leads to nitrate formation and not nitric acid production. Therefore, acid attack by these bacteria is unlikely [18,36]. Sulphur cycle bacteria (e.g. *Thiobacillus spp.*, *Thiothrix spp.*, *Beggiatoa spp.*) can convert limestone into gypsum, especially in polluted environments, which can lead to the formation of dark surface discolorations [18]. Sulphuric acid producers are, however, more readily found in building composites [36].

The aforementioned microorganisms typically thrive as a community, which develops in a succession. The order in which this succession occurs is still not consensual within the scientific community but photolithoautotrophic organisms may play an important role as primary colonizers, providing support for the posterior settlement of other organisms [5].

Considered by some authors [35] as the pioneering inhabitants of stone, algae are a diverse group of eukaryotic organisms that contain pigments such as chlorophyll, carotenoids and xanthophyll [37]. They share the spotlight with cyanobacteria, as they are both photosynthetic and can, therefore, establish a pioneer community on limestones and be fundamental in defining the primary bioreceptivity status. Once settled, they can support heterotrophic organisms (secondary bioreceptivity) [1] and promote the development of a self-feeding and more complex microbial community – biofilm. For this to happen, an extracellular matrix of proteins and sugar polymers is formed, increasing adherence and protecting the cells from desiccation while providing a greater surface area for it to expand [3,13,18].

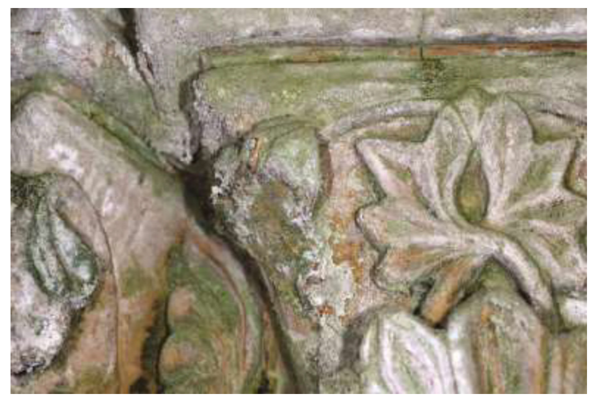

**Fig. 3.** Algae, bacteria and cyanobacteria are pioneer microorganisms which can cause significant colour alterations on stone, as displayed by the green and white shades seen above.

Besides being the most likely pioneer microorganisms and promoters of biofilm formation, algae and cyanobacteria also play a direct and important role in the physical and chemical deterioration of stone. This deterioration is characterized by the excretion of corrosive acids, especially on limestone and marble, and uptake/accumulation of sulphur, calcium and water into the cells causing cell enlargement and increased pressure on existing pores [38]. Colour alteration and the formation of black crust and patinas can also be expected [35]. Cyanobacteria also have the ability to survive under alternating cycles of drying and rehydration, and to protect themselves from UV radiation by producing protective pigments that cause colour alterations [35] (Fig. 3).

Still regarding community successions, it is important to note that the establishment of a heterotrophic community is possible even without the participation of phototrophs [18]. Heterotrophs can, for instance, use potential by-products of natural or synthetic substances applied during restoration treatments as carbon source, thus achieving a successful colonization [3,7,13,18]. Among these heterotrophs are *Fungi*, a group of chemoheterotrophic organisms characterized by filamentous hyphae. They lack the ability to synthesize their own food and, as such, cannot live unless organic compounds are present [37].

According to Sterflinger and Piñar [34], the erosive abilities of fungi may turn them into the worst enemy of stone as they can cause aesthetic, chemical, physical and mechanical deterioration, when the intrusion or penetration of fungal hyphae destabilizes the texture and structure of stone. In addition, fungi are acid producers that promote corrosion by weakening and dissolving the stone matrix during filamentous growth and colony development, since some of their hydrolytic enzymes are naturally degradative, and can cause discoloration [6]. The release of highly corrosive inorganic and organic acids, as well as chelating agents by fungi on stone are examples of processes involved in the promotion of biodeterioration [6,35], which may occur as a result of oxidative or reductive attack of reactive mineral constituents, such as manganese and iron [39].

Another possible stone contaminant are lichens, composite organisms that exhibit a symbiotic relationship between an algae or a cyanobacteria, and a fungi (mainly ascomycetes) [5,18,35,40]. The definition of lichen is, however, under study since recent finding have pointed to the presence of yeasts, a third element already being held accountable for some of the features displayed by the lichens [41]. There is still no data on whether this particular trio may also be present in limestone. Being highly resistant to desiccation and extreme temperatures they can thrive in hostile environments, and are frequently favoured by the presence bird droppings, rich in organic nitrogen [35,40,42]. Structures such as hyphae or rhizoids penetrate into wall pores assuring a better attachment and promoting the appearance of cracks and fissures, leading to structural and physical damage. Furthermore, the contraction-expansion cycles of lichens, consequence of desiccation and rehydration, can result in peeling and detachment of the upper mineral layer [18,35]. Their metabolic activities are often associated with the release of highly corrosive organic acids and chelating compounds, such as some of the lichenic acids produced [40], by which they form complexes with the mineral cations of the substratum. Nitrogen fixation abilities further improve this bioweathering potential. Their effects do not cease after their death as they leave behind a pitting corrosion due to the incorporation of mineral fragments into the thallus [35] which further increases stone susceptibility to biodeterioration. The symbiotic organisms that make up the structure of the lichen are often accompanied by their free-living forms [43], and both contribute to the mineral diagenesis and its dissolution [39]. This dissolution ability, displayed by both lichens and fungi, supplies vital nutrients to microbial communities allowing their survival and proliferation [39].

## 4. The Portuguese limestones and their microbial communities

In general, data regarding the microbial communities harboured by Portuguese monuments, most of them built of limestone or granite are scarce. In addition, it is worth mentioning that most of these studies are focused on specific organism types and their effects on a given substrate, in a symptom-directed approach [44,45] but there are no studies focusing on the total biodiversity of limestone. For instance, the review presented by Macedo et al. [8] focuses on both cyanobacteria and algae, whereas bacteria, fungi and lichens are not investigated Therefore, further studies are warranted in order to understand the biodiversity of the microbial colonization on Portuguese limestones, as well as the way they interact with each other and with the substrata.

The results gathered from published case-studies concerning the biological contamination of Portuguese limestone monuments and/or limestone based architectural features placed inside monuments, have been summarized in Table 1.

The level of taxonomic identifications reached in the studies summarized in Table 1 ranges from the species level to the phylum or even just the domain level. However, in all case-studies there was always an established community, likely initiated by phototrophic organisms (lichens, algae and cyanobacteria), and possibly followed by secondary colonization, in a succession supported by several authors [5–7,20,46]. Since the characteristics displayed by different stones influence the degree of biological contamination, a brief description of the different limestones is presented first, followed by a description of the corresponding contaminant microorganisms.

**Fig. 4.** Algae growing on a stone substrate where water is readily available. They form powdery or viscous layers and, as seen here, are responsible for dramatic chromatic changes.

All microorganisms mentioned in Table 1 exert some sort of mechanical and chemical stress on the stone material [6,7,35,42]. Furthermore, each of the taxa mentioned in the first column has been identified at least once and will be object of discussion in the following subsections of this manuscript.

### *4.1. Algae*

Most studies conducted in Portugal were aimed towards algae and cyanobacteria (Table 1). Among other biodeteriorative effects, as previously mentioned, these organisms are responsible for easily noticeable green and black stains [8,34] (Fig. 4).

According to Macedo et al. [8] the chlorophyta constitute the most common group of algae colonizing stone cultural heritage – *Chlorella* sp., *Myrmecia* sp., *Stichococcus* sp. and *Trebouxia* sp. were found in more than one location and type of limestone. Contrarily to other studies [8] chlorophyta were found in Portuguese dolomite. Regarding their biodeteriorative potential, microalgae such as *Cosmarium* sp., *Phormidium* sp. and *Symploca* sp. were considered major destructors of ornamental limestones collected from the fountain of Bibatauin (Granada, Spain) [35] but only *Cosmarium* sp. was identified in the studies presented in Table 1.

In fact, apart from the study performed in Granada, other international studies have only identified *Cosmarium* sp. in travertine stone [8]. *Klebsormidium flaccidum*, considered responsible for the formation of green and black sulphated crusts and biofilm in the Cathedral of Seville, was found on a biofilm in the Cathedral of Lund (Sweden) [35], and was also present in Portuguese limestone [8]. The genus *Chlorococcum*, considered one of the most common the Mediterranean Basin along with *Chlorella* and *Stichococcus*, is absent from the studies conducted in Portugal.

*Trebouxia* sp. was present in all enumerated lythotypes. These microalgae could be involved in the lichenization process, since it occurs in approximately 20 percent of all lichens and has rarely been found in free-living form [20].

It is important to note that the appearance and settlement of phototrophic communities is strongly dependent of external factors, such as exposure to light and water availability. In the same

**Table 1**
Studies on Portuguese limestone monuments and data released in terms of biodeterioration agents.

| | Stone type (monument) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ançã limestone | | | Ançã and Dolomitic limestone[a] | Lioz limestone | | | Unspecified limestone |
| | Sta. Cruz Church | | Alterpiece from Sé de Guarda | Sta-Clara-a-Velha Monastery | Ajuda National Palace | Belém Tower | Jeronimos Monastery | Convent of Christ, Tomar |
| Microorganisms (group, genus, species) | [8] | [16] | [44] | [20,51,52] | [20,52] | [75] | [43] | [67] |
| *Algae* | + | + | ND | + | – | + | ND | + |
| *Bracteococcus sp.* | + | – | | – | | – | | ND |
| *Chlorella sp.* | + | – | | + | | – | | |
| *Chlorella ellipsoidea* Gerneck | + | – | | – | | – | | |
| *Chlorella vulgaris* Beijerinck | – | – | | + | | | | |
| *Chlorokybus atmophyticus* Geitler | + | – | | – | | – | | |
| *Cosmarium sp.* | + | – | | – | | – | | |
| *Cylindrocystis brebissonii (Meneghini) De Bary* | + | – | | – | | – | | |
| *Desmococcus vulgaris Brand* | + | – | | – | | – | | |
| *Germinella terricola Petersen* | + | – | | – | | – | | |
| *Klebsormidium sp.* | + | – | | – | | – | | |
| *Klebsormidium flaccidum Kutzing, Silva, Mattox & Blackwell* | + | – | | – | | – | | |
| *Myrmecia sp.* | + | – | | + | | – | | |
| *Pleurastrum terrestre Fritsch & John* | + | – | | – | | – | | |
| *Podohedra bicaudata Geitler* | + | – | | – | | – | | |
| *Pseudococcomyxa simplex (Mainx) Fott* | + | – | | – | | – | | |
| *Scottiellopsis terrestris (Reisigl) Hanagata* | + | – | | – | | – | | |
| *Stichococcus sp.* | + for *bacillaris Nägeli* | + for *bacillaris Nägeli* | | + | | – | | |
| *Trebouxia sp.* | + | – | | + | | + | | |
| *Bacteria* | + | + | ND | + | + | + | + | + |
| Phylum *Cyanobacteria* | + | + | | + | + | + | + | ND |
| Order *Chroococcales* | + | + | | – | – | ND | + | |
| *Gloeocapsa sp.* | + | + for *alpina (Nägeli) Brand* | | – | – | | ND | |
| *Gloeocapsa novacekii* Kómarek & Anagnostidis e kuetzingiana Nägeli | + | – | | – | – | | ND | |
| *Chroococcidiopsis* | – | – | | – | + | | – | |
| *Cylindrospermopsis* | – | – | | – | + | | – | |
| *Leptolyngbya sp.* | – | – | | + | – | | – | |

Table 1 (*Continued*)

| | Stone type (monument) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ançã limestone | | | Ançã and Dolomitic limestone[a] | Lioz limestone | | | Unspecified limestone |
| | Sta. Cruz Church | | Alterpiece from Sé de Guarda | Sta-Clara-a-Velha Monastery | Ajuda National Palace | Belém Tower | Jeronimos Monastery | Convent of Christ, Tomar |
| Microorganisms (group, genus, species) | [8] | [16] | [44] | [20,51,52] | [20,52] | [75] | [43] | [67] |
| *Microcoleus* | – | – | | – | + | | – | |
| *Myxosarcina sp.* | + | – | | – | – | | – | |
| *Nostoc sp.* | + | – | | – | + | | – | |
| *Phormidium sp.* | + | – | | – | – | | – | |
| *Pleurocapsa* | – | – | | + | + | | – | |
| *Pseudocapsa* | – | – | | – | – | | + | |
| *Scytonema (sp.* and *javanicum* Bornet*)* | + | – | | – | – | | – | |
| *Tolypothrix sp.* | + | – | | – | – | | – | |
| Phylum *Proteobacteria* | ND | ND | | – | + | ND | ND | ND |
| *Methylobacterium* | | | | – | + | | | |
| Phylum *Acidobacteria* | ND | ND | | – | + | ND | ND | ND |
| Phylum *Actinobacter* | ND | ND | | + | – | ND | ND | ND |
| Cytophaga- Flavobacterium- bacteroidetes group | ND | ND | | + | – | ND | ND | ND |
| Phylum *Verrucomicrobia* | ND | ND | | + | – | ND | ND | ND |
| Fungi | ND | ND | + | + | ND | + | ND | + |
| *Aspergillus sp.* | | | – | – | | ND | | + |
| *Botrytis cinerea* | | | + | – | | | | – |
| *Capnobotryella sp.* | | | – | + | | | | – |
| *Cladosporium sp.* | | | + for *sphaerospermum* | + for *cladosporioides* | | | | + |
| *Cyphellophora lacciniata* | | | – | + | | | | – |
| *Engyodontium album* | | | + | – | | | | – |
| *Fusarium sp.* | | | – | – | | | | + |
| *Mucor sp.* | | | – | – | | | | + |
| *Penicillium sp.* | | | + *for frequentans* | – | | | | + |
| *Phoma sp.* | | | – | + | | | | – |
| *Rhizopus sp.* | | | – | – | | | | + |
| *Trichoderma viride* | | | + | – | | | | – |
| Lichens | ND | ND | ND | ND | ND | + | + | + |
| *Aspicilia sp.* | | | | | | – | + | ND |
| *Caloplaca sp.* | | | | | | + | + | |
| *Caloplaca aurantia* | | | | | | + | ND | |
| *Lecanora sp.* | | | | | | + | – | |
| *Squamarina crassa* | | | | | | + | – | |
| *Thyrea* | | | | | | – | + | |
| *Verrucaria* | | | | | | – | + | |
| *Xanthoria parietina* | | | | | | + | – | |

ND: not determined or not mentioned as having been explored; +: apart from the genera, a species was also confirmed.

[a] Due to its proximity to Coimbra, this dolomite is probably from the region of Anadia-Tomar, with a 9.5–18 percent MgO dolomite, high iron content and displays a yellow tone [21].

monument and lythotype one can, therefore, encounter different colonizing populations [47,48].

### 4.2. Bacteria

#### 4.2.1. Cyanobacteria

Cyanobacteria were identified in all of the mentioned case-studies. *Gloeocapsa*, *Nostoc* and *Pleurocapsa* were the only genera present in more than one location but *Gloeocapsa* was only identified in Ançã limestone.

According to Macedo et al. [8] limestone and marble display the greatest diversity of cyanobacteria and green algae within different lithotypes. The most frequently identified cyanobacteria belonged to the genera *Chroococcus*, *Gloeocapsa* (both from the order *Chroococcales*) and *Phormidium*, and they were considered ubiquitous in the Mediterranean basin [8]. *Gloeocapsa* sp. has been identified in painted surfaces, concrete, stone and tiles [38,49] while *Phormidium* sp. and *Chroococcus* sp. have already been retrieved from stone and concrete [38,46]. Both *Gloeocapsa* and *Phormidium* were present in the studies performed in Portuguese limestone (Table 1).

Many of the mentioned cyanobacteria (species from *Gloeocapsa*, *Phormidium*, *Scytonema*, *Lyngbya* and *Microcoleus*) frequently display a gelatinous sheath (sometimes pigmented) that acts as a water reservoir, which increases their resistance to extended drought periods as well as their adhesion to the substrate [20]. The *Pleurocapsa*-group and *Gloeocapsa*, as well as the filamentous genus *Scytonema*, are capable of causing discolouration and degrading the stone structure [50]. The genera *Nostoc*, *Tolypothrix* and *Gloeocapsa* are well-known for their ability to produce extracellular polymeric substances (EPS) that facilitate their attachment and survival in this relatively inhospitable environment [49]. Prior colonization by nitrogen-fixing cyanobacteria – such as *Cylindrospermopsis*, *Nostoc* and *Pleurocapsa* – is relevant for the establishment of other microorganisms, such as heterotrophic bacteria [20]. The presence of *Chroococcidiopsis*, the most desiccation-resistant cyanobacterium known [18] indicates that endolithic growth may occur in the analysed monuments [20].

It is likely that many other species are to be found in limestone supports if similar studies are performed in other monuments in a more comprehensive approach. Further studies on Portuguese limestones are warranted in order to perceive which cyanobacteria species/genera are more common in this particular setting and whether some of them are specific to this stone type, since this correlation has not yet been determined [8].

#### 4.2.2. Other bacteria

Domain *Bacteria* contains several phyla that are commonly found in stone, such as the phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* (a.k. *Cytophaga-Flavobacterium-Bacteroides* groups), as well as to the low GC firmicutes group [2]. Miller also identified the phylum *Verrucomicrobia* in Ançã limestone [20,51,52].

Most studies focusing on Portuguese limestones have pinpointed the presence of bacteria but lacked a thorough phylogenetic identification. For instance, Miller et al. [20,51,52] described the bacterial taxa found in Sta-Clara-a-Velha Monastery (Coimbra), although mostly only to the phylum level. The only genus presented in Table 1 – *Methylobacterium* – is a rhyzobial alphaproteobacteria which is characterized by its pink pigmentation and is included in the pink-pigmented facultative methylotrophs [53]. It has also been identified in other stone substrates [18,33,54] but its pigmentation has not been associated with any colour alteration in situ. Still on the phylum *Proteobacteria* are important chemolitotrophic bacteria, from the nitrogen and sulphur cycle, several of which have already been identified in stone [18]. Due to their important deterioration role, further and more in-depth studies are necessary on Portuguese stone (and limestone in particular).

Next in Table 1 are members of the phylum *Acidobacteria*, also found in the Ajuda National Palace. These are chemoorganotrophic organisms that have also been identified on stone [18] but the understanding of its potential contributions to its deterioration are still obscure.

The phylum *Actinobacteria*, found in dolomite (Table 1), has been associated with damaged limestone as some of the microorganisms included in this phylum promote a pH decrease and can, therefore, act as indicators of active biodeterioration [13]. Actinobacteria of the genera *Streptomyces*, *Micromonospora* and *Micropolyspora* show a high prevalence on decayed stone [18].

*Rubrobacter*, also an actinobacteria, has been appointed as an important causative agent of stone deterioration and discolouration [18,55,56]. Members of this genus have been widely described in disparate geographical locations as responsible for the rosy discoloration of wall paintings and stones, especially in salt-attacked monuments [26,57]. *Rubrobacter* can promote biofilm formation and hyphae penetration in painted layers, resulting in pitting, detachment, cracking and loss of paint [35]. Recently, a new species of *Rubrobacter* was identified in a Portuguese stone monument [56], which reinforces the relevance of performing such studies in limestone and other stones used in cultural heritage.

Besides these well-known examples, the phylum *Actinobacteri*a also harbours other genera, most of which were already identified in stone [18]. Members of the Actinobacteria phylum inhabit stone more effectively than most of the single-celled bacteria. This fact can be attributed to their filamentous growth and to their effective utilization of various nitrogen and carbon sources [58]. In addition, heat resistant actinobacteria are expected in temperate climates such as the Portuguese one [18]. The same can be expected for spore-forming bacteria because of their increased resistance. This is the case of the genus *Bacillus* (belonging to the phylum *Firmicutes* and not mentioned in Table 1), which is a common colonizer of stone buildings [13,18,28,59].

As happens with species of the genera Rubrobacter (Actinobacteria), Arthrobacter (Actinobacteria), Chromohalobacter (Actinobacteria), Myxococcus (Actinobacteria), Pseudomonas (Actinobacteria), Flavobacterium (Bacteroidete), some Bacillus spp. strains have also been reported to produce struvite, which suggests an active role in efflorescence niches and mineral precipitation [55]. In vitro experiments determined its ability to accelerate stone degradation [18] but no reference exists on the presence of this genera in Portuguese limestones, a clear sign of the need to further address this subject.

The phylum *Bacteroidetes* is a comprehensive group of *Bacteria* and its presence in stone has been mainly associated with halophilic species [28]. *Cytophaga* sp., *Rhodothermus* sp. and *Cyclobacterium* sp., have all been identified in a study on deteriorated wall paintings [54]. No further data is given on the findings presented in Table 1 regarding Sta. Clara-a-Velha Monastery.

Internationally, few comparisons have been performed between the bacterial contamination in the air and surfaces of cultural heritage sites. No Portuguese study exists on this subject but the influence that these two settings can have on each other may reveal itself of great interest since cross-contamination is more than likely to occur.

### 4.3. Fungi

In moderate or humid climates, fungal communities colonizing stone are mostly composed of common airborne fungi such as *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Aureobasidium*, *Phoma* and *Ulocladium.* These hyphomycetes establish their hyphal network through the stone pores [18,34]. However, in arid and semi-arid environments, such as those found in the Mediterranean area, the climatic conditions are too extreme for most hyphomycetes. In this case, the communities shift towards the so-called black yeasts and microcolonial fungi such as *Hortaea*, *Sarcinomyces*, *Coniosporium*, *Capnobotryella*, *Exophiala*, *Knufia* and *Trimmatostroma.* Microcolonial fungi are deeply embedded in the stone matrix being responsible for bio-pitting and are often in close association with lichens [34]. Given the strong presence of melanin in the cell walls of these fungi, they can completely cover a stone facade with a thick black layer [34,60] and while ignored until recently [61], the scientific community has gained interest on these major agents of stone decay.

Four of the Portuguese sites presented in Table 1 reported the presence of fungal contamination in limestone and in three of these the presence of common species found in rock [62], such as *Botrytis cinerea*, *Cladosporium sphaeospermum*, *Cladosporium cladosporioides* or *Engyodontium album* was confirmed. Other contaminants identified in stone were only identified to the genus level, as is the case for *Aspergillus*, *Capnobotryella*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus*.

Miller [20,51,52] used DNA sequence analysis to identify the fungal spectra present in Sta-Clara-a-Velha Monastery, and the techniques used allowed the identification of less common fungi (in stone or in the environment) such as *Cyphellophora lacciniata*. This example illustrates the benefits of incorporating modern analysis techniques in the field of stone biodeterioration.

In the review by Gadd [63] limestone appears to display a less diverse array of colonizing fungi when compared to other substrates such as granite, marble or sandstone. Only nine genera are considered common in this substrate and *Aspergillus*, *Engyodontium*, *Fusarium* and *Penicillium* are among them. Gadd points out that these records are probably very underestimated which highlights once more the need for further studies.

Closely related fungal taxa can present very diverse adaptive features; therefore, an in-depth analysis of the community is required in order to determine the actual culprits. The production of organic acids, for instance, is believed to play a major role in degradation of limestone; this effect varies between fungal species and different mineral substrates [63]. *Aspergillus fumigatus* can produce a green pigment and oxalic acid and is therefore particularly damaging to limestone; *Aspergillus niger*, also an oxalic acid producer, is well-known to degrade olivine, dunite, serpentine, muscovite, feldspar, spodumene, kaolin and nepheline [62]. *A. niger*, like many rock-dwelling dark pigmented fungi (such as *Penicillium simplissimum* and *Scopulariopsis brevicaulis*), can actively penetrate limestone and produce pits of up to 2 cm in diameter on rock surfaces [18,62,63].

The genus *Cladosporium* has been linked to damage not only in stone but also on the reinforcement substances used to protect it [35]. Along with *Penicillium*, *Fusarium*, *Phoma* and *Trichoderma*, this genus is also associated with acid secretion and bioweathering [35]; *Cladosporium sphaerospermum* (among other species) is a relevant melanin producer [62], as are *Alternaria alternata*, *Aureobasidium pullulans*, *Phoma glomerulata* or *Ulocladium chartarum*, among others [62]. *Botrytis*, alongside *Mucor*, *Penicillium* and *Trichoderma*, have shown the ability to solubilize calcium, magnesium and zinc silicates [63].

In the Cathedral of Évora (granite) the *Rhodotorula yeast* was implicated in the monument's rosy discoloration due to the carotenoid components of their metabolism. These substances are responsible for pink/orange spots that covered the wall surface and altered the original aspect of the stone [64]. *Rhodotorula* has been identified in international studies on rock surfaces other than limestone [39,62,63], but it was the first time its presence was documented in Portuguese monuments. Further studies on filamentous fungi and yeasts relate to limestone surfaces, air and surface analysis correlations, and state-of-the-art methods used to correctly identify fungi and yeasts will certainly improve the still handicapped knowledge on the matter.

### 4.4. Lichens

Lichens developing on stone are termed epilithic and, depending on the way these organisms attach to the stone, they are classified as crustose, foliose or fruticose, being this the succession in which they tend to appear [42]. Endolithic lichens only develop on calcareous materials, where they fully immerse their thallus on the stone matrix [40]. Being highly susceptible to pollution and the pH of stone [40], a protective function has also been appointed to them [65,66] specifically in the prevention of colonization by black fungi [6].

In Portugal, the study of colonization by lichens has been performed only on Lioz limestone in both the Belém Tower and Jerónimos Monastery (Table 1).

The presence of certain genera and lichen species is strongly related to light exposure and water availability [40]. *Caloplaca* sp., generally of the crustose type [42] was the only genera identified in more than one location. *Caloplaca ochracea* can assume an endolithic behaviour and is more prone to appear in shaded areas and cause biocorrosion, while *Caloplaca aurantia* (encountered in Belém Tower; Table 1) and *C. flavescens* have shown preference for sun-exposed areas. *Caloplaca citrina* favours the presence of nitrates on the stone surface [40].

Most of the lichens found in Portuguese limestone monuments (Table 1) are crustose, and these (along with the endolithic type) are also most capable of chemical and physical weathering [42] as their removal is particularly difficult since the thallus forms an strong physical connection to the substrate [18,40,66].

In a study performed on calcareous materials [65], the genera *Caloplaca* and *Xanthoria*, and the species *Diploicia canescens* and *Lecania rabenhorstii*, were identified. *Xanthoria* sp. was also found in the Belém Tower where the species *X. parietina* displayed a foliose-type thallus and a preference for sunlit surfaces [42].

As mentioned for *Caloplaca*, the genus *Lecanora* also shows differences according to species: *Lecanora muralis* has been shown to induce mechanical decay while *Lecanora dispersa* appeared harmless [40]. Genera *Aspicilia*, *Thyrea* and *Verrucaria* have all been identified in stone [18,42] and are all predominantly crustose in nature.

The potential for damage increases as communities evolve and grow in complexity. Studying, monitoring and acting when needed and desirable becomes imperative to better address this natural succession and its impending threats.

## 5. Common research methodology and techniques

As previously stated, knowledge of the lithotype and its state of conservation is very important to determine its primary bioreceptivity and to better assess the biodeterioration potential, as well as the methods needed to control it.

Chemical, mineralogical, petrographical and physical analyses are very relevant but, in fact, very few studies regarding biodeterioration and its key biological players have performed such assessments. Gravimetric analysis, colorimetry and atomic absorption spectrometry (AAS) can be useful to determine the chemical profiles of stone, while X-ray diffraction (XRD) and Fourier-transformed infrared spectroscopy (FTIR) can be used for mineral characterization [6,24]. Visual inspection using scanning electron microscopy (SEM/environmental SEM/back scattering EM – BSE) and thin section petrography are frequently used to analyse and characterize stone [6]. Regarding its porosity, fluid transport, open

**Table 2**
Methods used for the identification of microbial communities in the Portuguese studies on Portuguese limestone monuments (when disclosed).

| Methods | Portuguese studies (references) | | | | |
|---|---|---|---|---|---|
| | [44] | [20,51,52] | [75] | [43] | [67] |
| Optical microscopy (OM) | x | | | | |
| Scanning electron microscopy (SEM) | x | | | | |
| Scanning electron microscopy with back scattering (BS-SEM) | | | x | x | |
| Low temperature electron microscopy (LT-EM) | | | | x | |
| Transmission electron microscopy | | | | x | |
| Energy dispersive spectroscopy (EDS) | | | | x | |
| X-ray diffraction (XRD) | | | x | | |
| Conventional culture methods and OM | x | x | x | | x |
| Assimilation patterns and staining tests for identification of bacteria | | | | | x |
| Molecular biology protocols | | x | | | |

porosity and total porosity, the Hirschwald and capillarity coefficients are commonly assessed parameters [24].

For the identification of biological elements present in stone, the in situ visualization methods used to study the stone properties are also useful. The relation between the methods used to identify the microbial communities and the studies performed in Portuguese limestones is presented in Table 2.

Whenever samples are retrieved from stone supports, conventional culture methods, DNA analysis, or a combination of both methods are frequently used. Assimilation patterns are also an option [55,67]. From the data disclosed by the studies presented in Table 1, only Miller [20,51,52] used primers for bacteria and algae to perform direct identification, while the other studies used conventional culture methods only. In terms of bacterial species or genera, no conclusions were presented from the assimilation pattern and staining tests used in the study performed by Mateus et al. [67].

Conventional culture methods present the disadvantage of detecting just a small percentage of the real established community [2,67] a setback that can be minimized by selecting appropriate media for the study [6]. Halophilic and alkaliphilic Bacteria and *Archaea* taxa have been mainly identified using molecular biology methods since they are difficult to be cultivated [2] but this limitation can apparently be reduced when culture medium is supplemented with sodium chloride (ranging from 3–20% NaCl) [6,26,68]. The choice of adequate culture media is sometimes an issue by itself, since one risks privileging faster growing species [67] while overestimating their importance [59]. However, when combined with visual inspection it also provides the best insight into what is actively degrading the stone, while allowing further tests to be performed using the strains retrieved from the biodeteriorated location.

Molecular techniques enable to accurately elucidate the actual proportion of species present on a given environment. The fusion of cultivation and molecular strategies has delivered complementary results enabling a much better understanding of the taxonomy and diversity of the microorganisms inhabiting stone monuments [26,59]. Molecular techniques enable the study of microorganisms from their DNA, RNA and proteins. These techniques are developing at a fast pace and are complementing to more classical microbiological methods in the study of microorganisms. Even if these techniques have delivered reliable results of the microbial communities associated with stone materials, we still lack the ability to link most microorganisms to their metabolic roles in biodeterioration processes. New emerging technologies as next-generation sequencing (NGS) technologies have amazingly expanded the possibility of characterizing large populations of microbial communities co-existing in many different ecosystems and their interactions. In spite of these evidences, so far, NGS analyses have been scarcely used to analyse the microbial colonization of cultural assets, and especially stone monuments. Some examples of these few studies are the ones performed by Cutler et al. [69] who investigated algal and fungal communities on sandstone in the UK. Rosado et al. [70] investigated bacterial and fungal populations in mural paintings located in Portugal by combining culture-dependent methods and 454 pyrosequencing. Gutarowska et al. [71] performed NGS analysis of bacteria and fungi colonizing brick specimens of the historic buildings of Auschwitz in Poland. In addition, they employed state-of-the-art ultra-high performance liquid chromatography (UPLC) coupled to high-resolution mass spectrometry (HRMS), and time of-flight mass spectrometry (QToF-MS) allowing the detection of active metabolic pathways present in heritage materials. Chimienti et al. [72] performed NGS analyses of bacteria colonizing the stone of a Church in Italy. Dyda et al. [73] applied a metagenomics approach to select microbiological media to enable the most accurate determination of the bacterial diversity present in situ in microbiocenoses observed on historical stone objects. Finally, Adamiak et al. [30] used for the first time the Ion Torrent NGS platform to analyse the bacterial and archaeal communities present in brick and paint coatings. As mentioned, these are still scarce examples of the application of NGS analyses on cultural heritage, which nowadays can be easily applied in any kind of environment.

## 6. Concluding remarks and future perspectives

Based on the studies performed in Portuguese limestone monuments and presented in a summarized form in Table 1 most assessments were focused on Algae and Bacteria. Within the Algae, *Trebouxia* sp. was present in three different locations, while *Chlorella*, *Myrmecia* and *Stichococcus* genera were identified in two different monuments.

Within Bacteria, Cyanobacteria were always present in every study where these were sought, with *Gloeocapsa*, *Nostoc* and *Pleurocapsa* genera identified in more than one location. The remaining Bacteria have been under assessed in Portuguese studies. In fact, the present review on the biodeterioration of historic limestone monuments in Portugal highlights the need for further studies since the existing data is still very scarce on this extremely valuable and extensively used stone. International studies point to an existent biological diversity on stone materials that has not been described by the available studies performed in Portugal, which can be partially explained by the methodologies used so far. Improvements such as a wider selection of media and/or the application and further development of modern molecular biology protocols (such as NGS) can be invaluable in the detection of previously unidentified biodeterioration agents and in our ability to achieve a more complete picture of the complex communities found on limestone. Since being present does not necessarily translate into actual damage, future efforts should attempt to link taxa to the corresponding biodeterioration phenomena. Thus, apart from relying on genomics and classical culturing methods to ascertain communities, proteomic and metabolomic techniques may be applied in this field. Assessing the biological load in the environments surrounding historic stone landmarks may also prove useful to determine cross-contaminations. Other techniques, as Fourier-transformed infrared spectroscopy have been used in international studies [5,6,74] to fingerprint compounds left on the stone. RAMAN spectroscopy has also been useful in relating chromatic alterations to the presence of specific organisms [64] but these have not yet been used in Portuguese studies on limestone.

The survival and metabolic features that microbial communities display when colonizing stone can be, as presented, extremely deleterious for the conservation of historic monuments. In order to protect our cultural heritage – by either preventing or repairing damage – it is important to understand the interactions and microbial successions that can be established between colonizers and the stone matrix. Intrinsic factors, such as porosity and roughness, coupled with extrinsic environmental factors, are determinant to the establishment of a biological community. It is worth noting that prior conservation/restoration treatments and the present state of conservation are also relevant factors influencing the type of communities one can find in limestone and other stone types.

Given the different outputs, each analytical method can bring a comprehensive, multi-analytical approach. Combining stone analysis with conventional and molecular biology protocols should be the best approach to assess stone biodeterioration and to infer the appropriate corrective/conservative intervention.

## Acknowledgements

This work was financed by FEDER – Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 – Operational Programme for Competitiveness and Internationalisation (POCI), and by Portuguese funds through FCT – Fundação para a Ciência e a Tecnologia in the framework of the project POCI-01-0145-FEDER-PTDC/EPH-PAT/3345/2014. Nuno Mesquita was supported by POCH – Programa Operacional Capital Humano (co-funding by the European Social Fund and national funding by MCTES), with a Post-Doc Research grant (SFRH/BPD/112830/2015). Catarina Coelho was supported by Portuguese funds through FCT – Fundação para a Ciência e a Tecnologia in Project IN0756 – INV.EXPLORATORIA – IF/01061/2014. João Trovão was supported by POCH – Programa Operacional Capital Humano (co-funding by the European Social Fund and national funding by MCTES), through a "FCT – Fundação para a Ciência e Tecnologia" PhD research grant (SFRH/BD/132523/2017). Igor Tiago acknowledges an Investigator contract reference IF/01061/2014.

## References

[1] O. Guillitte, Bioreceptivity: a new concept for building ecology studies, Sci. Total Environ. 167 (1–3) (1995) 215–220.
[2] C.J. McNamara, R. Mitchell, Microbial deterioration of historic stone, Front. Ecol. Environ. 3 (8) (2005) 445–451.
[3] R.D. Wakefield, M.S. Jones, An introduction to stone colonizing microorganisms and biodeterioration of building stone, J. Eng. Geol. 31 (4) (1998) 369–373.
[4] P.S. Griffin, N. Indictor, R.J. Koestler, The biodeterioration of stone: a review of deterioration mechanisms, conservation case histories, and treatment, Int. Biodeterior. 28 (1–4) (1991) 187–207.
[5] T. Warscheid, J. Braams, Biodeterioration of stone: a review, Int. Biodeterior. Biodegrad. 46 (4) (2000) 343–368.
[6] C. Gaylarde, M. Ribas Silva, T. Warscheid, Microbial impact on building materials: an overview, Mater. Struct. 36 (5) (2003) 342–352.
[7] C.A. Crispim, C.C. Gaylarde, Cyanobacteria and biodeterioration of cultural heritage: A review, Microb. Ecol. 49 (1) (2005) 1–9.
[8] M.F. Macedo, A.Z. Miller, A. Dionísio, C. Saiz-Jimenez, Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview, Microbiology 155 (11) (2009) 3476–3490.
[9] C.C. Gaylarde, P.M. Gaylarde, B.A. Neilan, Endolithic phototrophs in built and natural stone, Curr. Microbiol. 65 (2) (2012) 183–188.
[10] N. Keshari, S.P. Adhikary, Diversity of cyanobacteria on stone monuments and building facades of India and their phylogenetic analysis, Int. Biodeterior. Biodegrad. 90 (2014) 45–51.
[11] P. Tiano, L. Addadi, S. Weiner, Stone reinforcement by induction of calcite crystals using organic matrix macromolecules. Feasability study, in: Proceedings of the 7th International Congress on Deterioration and Conservation of Stone, 1992, pp. 1317–1326.
[12] A.Z. Miller, N. Leal, L. Laiz, R. Candelera, M.A. Silva, A. Dionisio, M.F. Macedo, C. Sáiz-Jiménez, Primary bioreceptivity of limestones used in southern European monuments, Geol. Soc. London 331 (2010) 79–92.
[13] P.J.A. Skipper, L.K. Skipper, A survey of bacterial colonisation of historic limestone buildings: Lincoln Cathedral and St. Peter at Gowts, United Kingdom, Rehab 2014, in: Proceedings of the International Conference on Preservation, Maintenance and Rehabilitation of Historic Buildings and Structures, 2014, pp. 1003–1012.
[14] C.C. Gaylarde, P.M. Gaylarde, I.B. Beech, Deterioration of limestone structures associated with copper staining, Int. Biodeterior. Biodegrad. 62 (2) (2008) 179–185.
[15] A.Z. Miller, P. Sanmartín, L. Pereira-Pardo, A. Dionisio, C. Saiz-Jimenez, M.F. Macedo, B. Prieto, Bioreceptivity of building stones: a review, Sci. Total Environ. 426 (2012) 1–12.
[16] A. Miller, A. Dionísio, M.F. Macedo, Primary bioreceptivity: a comparative study of different Portuguese lithotypes, Int. Biodeterior. Biodegrad. 57 (2) (2006) 136–142.
[17] Ž. Savković, N. Unković, M. Stupar, M. Franković, M. Jovanović, S. Erić, K. Šarić, S. Stanković, I. Dimkić, J. Vukojević, M. Ljaljević Grbić, Diversity and biodeteriorative potential of fungal dwellers on ancient stone stela, Int. Biodeterior. Biodegrad. 115 (2016) 212–223.
[18] S. Scheerer, O. Ortega-Morales, C. Gaylarde, Microbial deterioration of stone monuments – An updated overview, Adv. Appl. Microbiol. 66 (2009) 97–139.
[19] Aragonite, Mineral. Soc. Am. 2 (1) (2005) (Chantilly, VA 20151-1110) http://www.handbookofmineralogy.org/ http://www.handbookofmineralogy.org/pdfs/calcite.pdf http://www.handbookofmineralogy.org/pdfs/aragonite.pdf.
[20] A.Z.A.F. Miller, Primary bioreceptivity of limestones from the Mediterranean basin to phototrophic microorganisms, 2010.
[21] G. Manupella, J.C. Moreira, M.L. Romão, Panorama dos Dolomitos e Calcários Dolomíticos Portugueses, Bol. Minas do Inst. Geol. e Min. 4 (1981) 3–15.
[22] J. Carvalho, G. Manuppella, A.C. Moura, Calcários ornamentais portugueses, Bol. Minas do Inst. Geol. e Min. 37 (4) (2001) 223–232.
[23] L. Aires-Barros, L. Picciochi Alves, Estudo sobre o estado de alteração de alguns monumentos portugueses, Bol. da Soc. Port. Química 28 (II) (1987) 59–63.
[24] C.A.M. Figueiredo, L. Aires-Barros, M.J. Basto, R.C. Graca, A. Mauricio, The weathering and weatherability of Basilica da Estrela stone, Lisbon, Portugal, Build. Stone Decay (2007) 99–107.
[25] C. Figueiredo, L. Aires-Barros, M.J. Neto, The church of Santa Engracia (the National Pantheon, Lisbon, Portugal): building campaigns, conservation works, stones and pathologies Limestone in the Built Environment: Present-Day Challenge for the Preservation of the Past, vol. 331, B. Smith, G.-H.M.,H. Viles, J. Cassar, London, 2010, pp. 183–193.
[26] J. Ettenauer, K. Sterflinger, G. Piñar, Cultivation and molecular monitoring of halophilic microorganisms inhabiting an extreme environment presented by a salt-attacked monument, Int. J. Astrobiol. 9 (01) (2010) 59–72.
[27] G. Pinar, C. Saiz-Jimenez, C. Schabereiter-Gurtner, M.T. Blanco-Varela, W. Lubitz, S. Rölleke, Archaeal communities in two disparate deteriorated ancient wall paintings: detection, identification and temporal monitoring by denaturing gradient gel electrophoresis, FEMS Microbiol. Ecol. 37 (1) (2001) 45–54.
[28] G. Piñar, K. Sterflinger, J. Ettenauer, La vie en rose: a review of the rosy discoloration of subsurface monuments, in: C Saiz-Jimenez (Ed.), The Conservation of Subterranean Cultural Heritage, Taylor & Francis, London, 2014, pp. 113–124.
[29] G. Piñar, K. Ripka, J. Weber, K. Sterflinger, The micro-biota of a subsurface monument the medieval chapel of St. Virgil (Vienna, Austria), Int. Biodeterior. Biodegrad. 63 (7) (2009) 851–859.
[30] J. Adamiak, A. Otlewska, H. Tafer, K. Lopandic, B. Gutarowska, K. Sterflinger, G. Piñar, First evaluation of the microbiome of built cultural heritage by using the Ion Torrent next-generation sequencing platform, Int. Biodeterior. Biodegrad. (2017) 11–18, http://dx.doi.org/10.1016/j.ibiod.2017.01.040.
[31] S. Castanier, G. Le Métayer-Levrel, J.P. Perthuisot, Ca-carbonates precipitation and limestone genesis – the microbiogeologist point of view, Sediment. Geol. 126 (1–4) (1999) 9–23.
[32] G. Piñar, C. Jimenez-Lopez, K. Sterflinger, J. Ettenauer, F. Jroundi, A. Fernandez-Vivas, M.T. Gonzalez-Muñoz, Bacterial community dynamics during the application of a Myxococcus xanthus-inoculated culture medium used for consolidation of ornamental limestone, Microb. Ecol. 60 (1) (2010) 15–28.
[33] J. Ettenauer, G. Piñar, K. Sterflinger, M.T. Gonzalez-Muñoz, F. Jroundi, Molecular monitoring of the microbial dynamics occurring on historical limestone buildings during and after the in situ application of different bio-consolidation treatments, Sci. Total Environ. 409 (24) (2011) 5337–5352.
[34] K. Sterflinger, G. Piñar, Microbial deterioration of cultural heritage and works of art – Tilting at windmills? Appl. Microbiol. Biotechnol. 97 (22) (2013) 9637–9646.
[35] T. Dakal, S. Cameotra, Microbially induced deterioration of architectural heritages: routes and mechanisms involved, Environ. Sci. Eur. (2012) 24–36.
[36] P. Gaylarde, C. Gaylarde, Deterioration of siliceous stone monuments in Latin America: microorganisms and mechanisms, Corros. Rev. 22 (5–6) (2004) 395–416.
[37] S. Jayakumar, Studies on the Biodeterioration of Concrete by Marine Algae, Department of Civil Engineering, Pondicherry University, India, 2009.

[38] S. Vijayakumar, Role of Cyanobacteria in biodeterioration of historical monuments – A review, BMR Microbiol. 1 (1) (2014) 1–13.
[39] E.P. Burford, M. Kierans, G.M. Gadd, Geomycology: fungi in mineral substrata, Mycologist 17 (3) (2003) 98–107.
[40] M. Lisci, M. Monte, E. Pacini, Lichens and higer plants on stone: a review, Int. Biodeterior. Biodegrad. 51 (2003) 1–17.
[41] T. Spribille, V. Tuovinen, P. Resl, D. Vanderpool, H. Wolinski, M.C. Aime, K. Schneider, E. Stabentheiner, M. Toome-Heller, G. Thor, H. Mayrhofer, H. Johannesson, J.P. McCutcheon, Basidiomycete yeasts in the cortex of ascomycete macrolichens, Science 353 (6298) (2016) 488–492.
[42] J. Garcia-Rowe, C. Saiz-Jimenez, Lichens and bryophytes as agents of deterioration of building materials in Spanish cathedrals, Int. Biodeterior. 28 (1–4) (1991) 151–163.
[43] C. Ascaso, J. Wierzchos, V. Souza-Egipsy, A. De Los Ríos, J.D. Rodrigues, In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon), Int. Biodeterior. Biodegrad. 49 (1) (2002) 1–12.
[44] J. Girbal, M. Iglesias, J.L. Prada, M. Pugés, R. Rocabayera, Estudio de las patologias del Retablo del Altar Mayor de la Sé da Guarda, Estud. Patrim. 3 (2002) 70–78.
[45] A.Z. Miller, A. Dionísio, L. Laiz, M.F. Macedo, C. Saiz-Jimenez, The influence of inherent properties of building limestones on their bioreceptivity to phototrophic microorganisms, Ann. Microbiol. 59 (4) (2009) 705–713.
[46] C.C. Gaylarde, L.H.G. Morton, Deteriogenic biofilms on buildings and their control: a review, Biofouling 14 (1) (1999) 59–74.
[47] C. Hallmann, L. Stannek, D. Fritzlar, D. Hause-Reitner, T. Friedl, M. Hoppert, Molecular diversity of phototrophic biofilms on building stone, FEMS Microbiol. Ecol. 84 (2) (2013) 355–372.
[48] Q. Li, B. Zhang, Z. He, X. Yang, Distribution and diversity of bacteria and fungi colonization in stone monuments analyzed by high-throughput sequencing, PLoS One 11 (9) (2016) 1–17.
[49] M.L. Coutinho, et al., Microbial communities on deteriorated artistic tiles from Pena National Palace (Sintra, Portugal), Int. Biodeterior. Biodegrad. 84 (2013) 322–332.
[50] C.A. Crispim, P.M. Gaylarde, C.C. Gaylarde, B.A. Neilan, Deteriogenic cyanobacteria on historic buildings in Brazil detected by culture and molecular techniques, Int. Biodeterior. Biodegrad. 57 (4) (2006) 239–243.
[51] A.Z. Miller, L. Laiz, J.M. Gonzalez, A. Dionísio, M.F. Macedo, C. Saiz-Jimenez, Reproducing stone monument photosynthetic-based colonization under laboratory conditions, Sci. Total Environ. 405 (1–3) (2008) 278–285.
[52] A.Z. Miller, L. Laiz, A. Dionísio, M.F. Macedo, C. Saiz-Jimenez, Growth of phototrophic biofilms from limestone monuments under laboratory conditions, Int. Biodeterior. Biodegrad. 63 (7) (2009) 860–867.
[53] D. Balachandar, P. Raja, S.P. Sundaram, Genetic and metabolic diversity of pink-pigmented facultative methylotrophs in phyllosphere of tropical plants, Brazilian J. Microbiol. 39 (1) (2008) 68–73.
[54] C. Gurtner, J. Heyrman, G. Piñar, W. Lubitz, J. Swings, S. Rölleke, Comparative analyses of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis, Int. Biodeterior. Biodegrad. 46 (3) (2000) 229–239.
[55] L. Laiz, A.Z. Miller, V. Jurado, E. Akatova, S. Sanchez-Moral, J.M. Gonzalez, A. DionÌsio, M.F. Macedo, C. Saiz-Jimenez, Isolation of five Rubrobacter strains from biodeteriorated monuments, Naturwissenschaften 96 (1) (2009) 71–79.
[56] V. Jurado, A.Z. Miller, C. Alias-Villegas, L. Laiz, C. Saiz-Jimenez, Rubrobacter bracarensis sp. nov., a novel member of the genus Rubrobacter isolated from a biodeteriorated monument, Syst. Appl. Microbiol. 35 (5) (2012) 306–309.
[57] C. Schabereiter-Gurtner, G. Piñar, D. Vybiral, W. Lubitz, S. Rölleke, Rubrobacter-related bacteria associated with rosy discolouration of masonry and lime wall paintings, Arch. Microbiol. 176 (5) (2001) 347–354.
[58] M. Saarela, H.L. Alakomi, M.L. Suihko, L. Maunuksela, L. Raaska, T. Mattila-Sandholm, Heterotrophic microorganisms in air and biofilm samples from Roman catacombs, with special emphasis on actinobacteria and fungi, Int. Biodeterior. Biodegrad. 54 (1) (2004) 27–37.
[59] L. Laiz, G. Piñar, W. Lubitz, C. Saiz-Jimenez, Monitoring the colonization of monuments by bacteria: cultivation versus molecular methods, Environ. Microbiol. 5 (1) (2003) 72–74.
[60] O. Cuzman, R. Olmi, Preliminary study on controlling black fungi dwelling on stone monuments by using a microwave heating system, Int. J. Conserv. Sci. 4 (2) (2013) 133–144.
[61] S. Onofri, L. Zucconi, D. Isola, L. Selbmann, Rock-inhabiting fungi and their role in deterioration of stone monuments in the Mediterranean area, Plant Biosyst. - An Int. J. Deal. Asp. Plant Biol. 3504 (2014) 1–8.
[62] K. Sterflinger, Fungi as geologic agents, Geomicrobiol. J. 17 (2) (2000) 97–124.
[63] G.M. Gadd, Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation, Mycol. Res. 111 (1) (2007) 3–49.
[64] T. Rosado, A. Reis, J. Mirão, A. Candeias, P. Vandenabeele, A.T. Caldeira, Pink! Why not? On the unusual colour of Évora Cathedral, Int. Biodeterior. Biodegrad. 94 (2014) 121–127.
[65] C. Ascaso, J. Wierzchos, R. Castello, Study of the biogenic weathering of calcareous litharenite stones caused by lichen and endolithic microorganisms, Int. Biodeterior. Biodegrad. 42 (1) (1998) 29–38.
[66] D. Pinna, Biofilms and lichens on stone monuments: do they damage or protect? Microbiotechnol., Ecotoxicol. Bioremediation – Front. Microbiol. 5 (2014) 133–136.
[67] D.M.R. Mateus, R.B. Silva, F.M.C. Costa, J.P.F. Coroado, Diversidade microbiológica do edifício da Sacristia Incompleta do Convento de Cristo, em Tomar, e avaliação do seu controlo por biocidas, Conserv. Património 17 (2013) 11–20.
[68] G. Piñar, L. Kraková, D. Pangallo, D. Piombino-Mascali, F. Maixner, A. Zink, K. Sterflinger, Halophilic bacteria are colonizing the exhibition areas of the Capuchin Catacombs in Palermo, Italy,î, Extremophiles 18 (4) (2014) 677–691.
[69] N.A. Cutler, A.E. Oliver, H.A. Viles, S. Ahmad, A.S. Whiteley, The characterisation of eukaryotic microbial communities on sandstone buildings in Belfast, UK, using TRFLP and 454 pyrosequencing, Int. Biodeterior. Biodegrad. 82 (2013) 124–133.
[70] T. Rosado, J. Mirão, A. Candeias, A.T. Caldeira, Microbial communities analysis assessed by pyrosequencing – A new approach applied to conservation state studies of mural paintings, Anal. Bioanal. Chem. 406 (3) (2014) 887–895.
[71] J. Skóra, B. Gutarowska, K. Pielech-Przybylska, L. Stępień, K. Pietrzak, M. Piotrowska, Assessment of microbiological contamination in the work environments of museums, archives and libraries, Aerobiologia (Bologna) 31 (3) (2015) 389–401.
[72] G. Chimienti, R. Piredda, G. Pepe, I.D. van der Werf, L. Sabbatini, C. Crecchio, P. Ricciuti, A.M. D'Erchia, C. Manzari, G. Pesole, Profile of microbial communities on carbonate stones of the medieval church of San Leonardo di Siponto (Italy) by Illumina-based deep sequencing, Appl. Microbiol. Biotechnol. 100 (19) (2016) 8537–8548.
[73] M. Dyda, P. Decewicz, K. Romaniuk, M. Wojcieszak, A. Sklodowska, L. Dziewit, L. Drewniak, A. Laudy, Application of metagenomic methods for selection of an optimal growth medium for bacterial diversity analysis of microbiocenoses on historical stone surfaces, Int. Biodeterior. Biodegrad. (2017) 1–9, http://dx.doi.org/10.1016/j.ibiod.2017.03.009.
[74] G. Gómez-Alarcón, B. Cilleros, M. Flores, J. Lorenzo, Microbial communities and alteration processes in monuments at Alcala de Henares, Spain, Sci. Total Environ. 167 (1995) 231–239.
[75] C. Ascaso, J. Wierzchos, J.D. Rodrigues, L. Aires-Barros, F.M. Henriques, E.E. Charola, Endolithic microorganisms in the biodeterioration of the tower of Belem, Int. Z. Bauinstandsetzen 4 (1998) 627–640 (Retrieved from http://digital.csic.es/handle/10261/33048, ).