"Air and wall mycobiota interactions - a case study in the Old Cathedral of Coimbra"

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Abstract

The microbiota present in public buildings - fungal, algae and fungi that thrive in buildings and in their construction materials - influence the structural condition as well as, potentially, the health of those who live, work, or visit them. These organisms can colonise and deteriorate all kinds of construction materials such as stone, wood, bricks, glass, steal and metals, concrete, ceramics, tiles, among others. One of the vehicles that helps to spread and therefore contributes to this biological contamination is the air and its microbiome in such environments.
In this work we analysed the fungal air burden existing in the cloister of the Old Cathedral of Coimbra, in four chapels and the central square of this cloister, in two different seasons. This allowed relating the fungal air burden with the established fungal communities (mycobiota) that were present in biodeteriorated spots on the walls of the studied chapels, in the context of a previous work from our research team.
The fungal air burden was higher in the summer, although with lower diversity. Patterns of distribution varied between sites, but in general, the most abundant species were found present in both the central square and chapels, suggesting that the air flows between these places are likely to vector the exchange of fungal propagules. Moreover, some less frequent species were found specific to particular chapels, and were not found in the air samples from the central square.
These findings support the idea of the specificity and environmental requirements of most retrieved isolates, while showing that the chapels have the potential to host a large set of organisms that are not present elsewhere. Many of these fungi are linked to biodeterioration phenomena of the walls and/or are associated to pathogenic and toxigenic effects in humans.
This study highlights the relevance of assessing the microbiota that thrive in such settings, and how the design and architecture can influence the composition of the established microbiota.

Keywords: Fungal aerosols, Air sampling, Cultural Heritage, Biodeterioration, Micobiome

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X.1 – Introduction

For many centuries stone has been a key element to humankind, used for construction, tool making, artworks and many other ends. Its high durability and ubiquitous availability made it the material of choice for our ancestors, a trend that thrives to this day.

However, many other organisms share our interest with this material. Photosynthetic microorganisms and algae are frequently the first colonizers of stone buildings, and after their establishment, they will provide the nutrients required for bacterial and fungal growth. Each of these organisms has a set of abilities that allow them to endure, and consequently damaging the stone substrate through physical and chemical methods. The combined actions of these colonizers can lead to chemical shifts in the composition of stone, disaggregation, aesthetic damages, loss of information, and, ultimately, pose a threat to the preservation of important historic and artistic landmarks (Ortega-Morales et al., 1991; Liu et al., 2020; Prieto et al., 2020). Their development on stone is conditioned by resource availability, physical structure (e.g., texture and porosity) and mineral composition of the support (Papida et al., 2000; Gleeson et al., 2005; Guillite, 1995; Prieto and Silva, 2005; Miller et al., 2006)

Biodeterioration can be defined as a set of undesirable changes in the properties of a valuable material caused by the complex physical and chemical actions of living organisms, and a generally undesired breakdown of materials by microbial action. These phenomena are particularly relevant when it comes to cultural heritage, monuments, sculptures, and statues, that require expensive preventive and conservative actions (Wakefield and Jones, 1998), and carbonate rocks such as limestones, given their high porosity, softness, and intrinsic bioreceptivity, are particularly prone to these

occurrences (Miller et al., 2006, 2010; Miller, 2010; Gadd 2017, Trovão et al., 2019; Soares et al., 2019).

Fungi are considered the worst enemy of stone, as they can promote chemical, physical, mechanical, and aesthetic deteriorations (Sterflinger and Piñar, 2013). They are ubiquitous heterotrophic organisms that withstand extreme environmental conditions, by adopting different morphologic and metabolic strategies, and this allows them to successfully colonize stone, wood, metal and other materials (Scheerer et al., 2009; Sterflinger and Piñar, 2013; Gadd, 2017a, b). From an ecological standpoint, lithobiontic fungi (i.e., fungi that can thrive on rocks and stone) can develop on the surface of the stone (epilithic) or on stone pores and fissures (endolithic). Filamentous fungi are more frequent in moderate and humid climates, whilst microcolonial black fungi (MCF) tend to be dominant in arid and semi-arid environments, due to their extremophile characteristics and adaptations, salt tolerance, and resistance to desiccation (Selbmann et al., 2015). Their successful growth contributes to several biodeterioration phenomena, such as epilithic stains, dark crusts, salt efflorescences, discoloration, biopitting, and biofilm development (Cuzman et al., 2011).

During filamentous growth and colony development, fungi can induce the corrosion and degradation of stone through the dissolution of its mineral matrix, by releasing highly corrosive inorganic and organic acids, metabolites, exoenzymes, and metal chelating agents, often present in extracellular mucilaginous substances (Burford et al., 2003; Scheerer et al., 2009; Gadd and Raven, 2010).

An additional effect in carbonate substrates is fungal stone diagenesis (i.e., the dissolution and the possible substitution of the original stone minerals by secondary biomineralization) by fungal mediated secondary biomineralization (Gadd, 2007, 2017a, b). The formation of different oxalates is common in the decay and spoilage of rock, mineral-based structures, and building components such as heritage stone, concrete, cement, mortars, and others (Dakal and Cameotra, 2012). Oxalic acid is produced by a wide variety of fungal organisms as a natural by-product of their metabolism, including saprotrophic, symbiotic and pathogenic species (Gadd et al., 2014) and is a strong solubilizing agent (Sterflinger, 2000). A comprehensive review on oxalate-producing fungi dates back to 1996 (Dutton and Evans, 1996), and is still a reference today. Oxalic acid excreted by fungal organisms dissolves calcium carbonate, the key component of the limestone matrix, and reacts with free Ca^{2+} (Sterflinger, 2000). It is widely found in patinas on the surface of limestone monuments as the formation of calcium oxalates in the forms of whewellite and weddellite, and this process is considered one of the most relevant weathering processes affecting historic monuments (Gadd et al., 2014).

They can also take part in discoloration processes (i.e., pigmentation and staining of the substrate) because of different melanin types present in their cells and hyphae, and through their involvement in the development of subaerial biofilms. Finally, physical erosion through the osmotic pressure effected during hyphal growth is also a concern, as it promotes disaggregation and flaking on stone (Sterflinger, 2000; Gaylarde et al., 2003; Sterflinger, 2010; Dakal and Cameotra, 2012; De Leo et al., 2019; Trovão et al., 2020).

The surrounding environment is one of the paths that organisms use to reach the substrate. Aerobiological studies characterize the biological aerosol in terms of quantity and quality of present organisms, to assess the risks to materials and human health, and assist in the development of strategies to help minimize these issues. The settlement of these bio aerosols on stone-made historical buildings represents a serious biodeterioration concern, since they may grow on these materials once adequate conditions (i.e., water, temperature, nutrients, etc.) are favourable, and eventually contribute to their biodeterioration (Caneva et al., 2007, 2020; Paiva de Carvalho et al., 2018; Savković et al., 2021).

The toxigenic potential of some organisms of these organisms and their effects on human health is also a topic of concern. Due to the production of mycotoxins, they are frequently associated with respiratory disorders, as is the case with some species from genera *Alternaria, Aspergillus,* and *Stachybotrys* (WHO, 2009, Baxi et al., 2016). The public health and the exposure of staff members and visitors are relevant matters, where both the quantity and quality of these organisms are key to assure a healthy environment – a characteristic that is often overlooked in cultural heritage.
One method for the retrieval of viable propagules from the air is through the use of volumetric air samplers, that pull a pre-determined volume of air and direct it through a set of pores, into a culture plate with a solid growth medium. When propagules impact the culture medium, they stick and grow on the surface. It is then possible to count the forming colonies and calculate their concentration in the air (usually expressed in CFU/m^3 (Colony-Forming Units per cubic meter of air)). The use of different culture media (i.e., Malt Extract Agar, Potato Dextrose Agar, Dichloran-Glycerol agar), broadens the range of retrievable organisms, since there are specific nutrient requirements or different growth rates to take into account. After their growth, one can perform their morphological and molecular-based identification, via the amplification and sequencing of taxa-specific genes. That information is then used to determine the qualitative and quantitative composition of the different species that are present in the air.

The site “University of Coimbra – Alta and Sofia” was granted the World Heritage status by UNESCO in 2013, raising the awareness and the commitment necessity for the preservation of this patrimony. Included in the classified area are important limestone-built monuments, which currently display different signs of biodeterioration. One of these monuments, maybe the most emblematic, and one of the most visited monuments in the city of Coimbra (Portugal) is the Old Cathedral of Coimbra – also known as Sé Velha (Figure 1).

This cathedral is a Romanesque church built between the XII and XIII centuries, using soft and porous limestone (Ançã and Portunhos (Portugal)) and dolomite, both particularly prone to deterioration by weather conditions, pollution, and the established microbiological communities (Miller et al., 2010; Miller, 2010; Soares et al., 2019, Trovão et al., 2019).

Fig 1 – The Old Cathedral of Coimbra

The Ançã limestone is a homogenous, finely grained, pure, bright limestone with a relative weight proportion of $CaCO_3$ superior to 96.5%. Due to its low compressive strength and hardness, it was extensively used in Portuguese monuments. Dolomitic limestone is composed of both $CaCO_3$ and dolomite, a calcium magnesium carbonate mineral ($CaMg(CO_3)_2$). A yellow-toned dolomite (low magnesium and high iron content) has been extensively used in the region of Coimbra, usually extracted from local quarries (Manupella et al., 1981; Aires-Barros and Alves, 1987; Mauricio, 2001; Miller, 2010; Miller et al., 2010; Catarino et al., 2019).

Figure 2 – The Cloister of the Old Cathedral of Coimbra

The studied monument hosts a cloister (Figure 2), that is located at the south side of the church and is comprised of four semi-open chapels (Figures 3 and 4) that are in contact with the exterior environment, with no physical barriers separating them from the exterior.

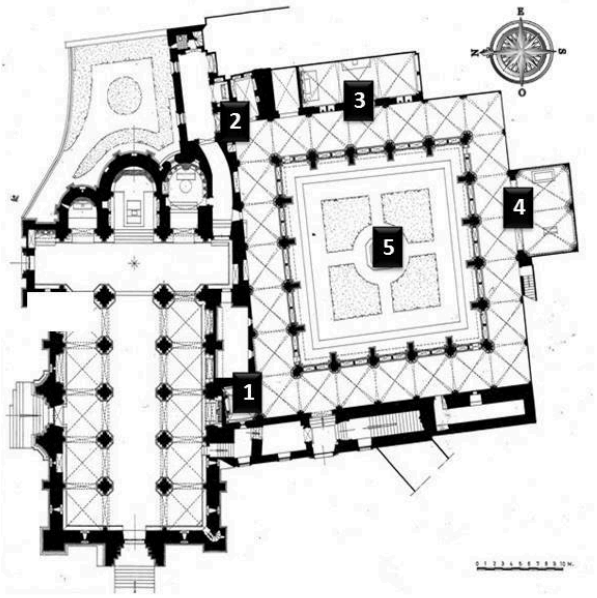

Figure 3 - The Old Cathedral of Coimbra ("Sé Velha") – The cloister and its chapels.
(1 - Natividade; 2 - São Miguel; 3 - Santa Maria; 4 - São Nicolau; 5 - Exterior)
(Adapted from the website "Sistema de Informação para o Património Arquitectónico")

Some of these chapels display several signs of abiotic and biotic promoted forms of deterioration, in the form of dark discolorations, different biofilms in the stone surfaces as well as salt efflorescences, all of which are severely affecting the limestone walls of this monument, both structurally and aesthetically. The presence of water that is almost constantly running down some of its walls in the interior cloister is a major problem, as it promotes the establishment and sustains the development of biofilms, but also because it allows salt mobilization (gypsum) to the surface, characterized by salt efflorescences.

It is known that tourism and frequent visits to monuments have a considerable role and influence in the biodeterioration of stone-made buildings, as anthropogenic activity is a vector for the dissemination of fungal organisms. Caust and Vecco (2017) state that the quick growth in cultural tourism poses serious concerns about the cultural and environmental integrity of cultural destinations, even more so for World Heritage sites. Preliminary tests for the removal of existing microbial communities have been carried out, without a deep understanding of neither the established communities nor the underlying effects of interfering with these populations. It is possible in such cases, that these interventions end up doing more harm than good in the long term, by allowing the recolonization by other, potentially more harmful microbial populations (Scheerer, 2008; Doehne and Price, 2010; Caust and Vecco, 2017). To address this, several studies have been recently conducted in this monument, focusing on the deep characterization

of the structural diversity of the fungal (Trovão et al., 2019), algal (Soares et al., 2019), and bacterial (Coelho et al., 2021) communities from biodeteriorated spots from the stone walls of these chapels.

The purpose of our work was to characterize this environment in what concerns the airborne fungal taxa, and to compare it with the established fungal communities, already described and characterized in this location by Trovão et al. (2019), to better understand the relevance of this interface, and how air and stone populations relate. This study focused on the culturable fraction of fungal organisms, and even though this process doesn't allow to retrieve all the organisms present in the air (since it fails to detect inactive (viable, but non-culturable), or non-viable forms (Dakal and Arora, 2012; Otlewska et al., 2014; Mihajlovski et al., 2015; Sanmartín et al., 2016)), it does allow the characterization of most of the viable and active organisms, and it is the usual approach when analyzing air samples.

Furthermore, regardless of being an outdoor or indoor location, air samples are relevant reference tools in determining the fungal bioburden, to allow pinpointing the most relevant species in this site, in what concerns biodeterioration as well as human health. Finally, since weather, climatic changes, air pollution, mycobiomes, and stone conservation are intertwined, these should all be considered when discussing bio receptivity and especially conservation strategies for a given heritage building as the Old Cathedral of Coimbra (Warscheid and Braams, 2000; Miller et al., 2012).

The aims of this study were to:

- Assess the diversity of fungal organisms present in the air of the cloister exterior and its four chapels (in two seasons).
- Relate species isolated from the open area of the cloister and the different chapels, to understand the influence of natural airflow as a vector for dissemination.
- Relate identified species with species previously retrieved from particular biodeteriorated spots in limestone walls (Trovão et al., 2019).
- Pinpoint species with potential pathogenic and toxigenic effects to humans.

X.2 – Materials and Methods

X.2.1 – Sampling Sites

Air samples were collected inside the four chapels (Figure 4) and the exterior of the cloister (Central square, Figure 2).

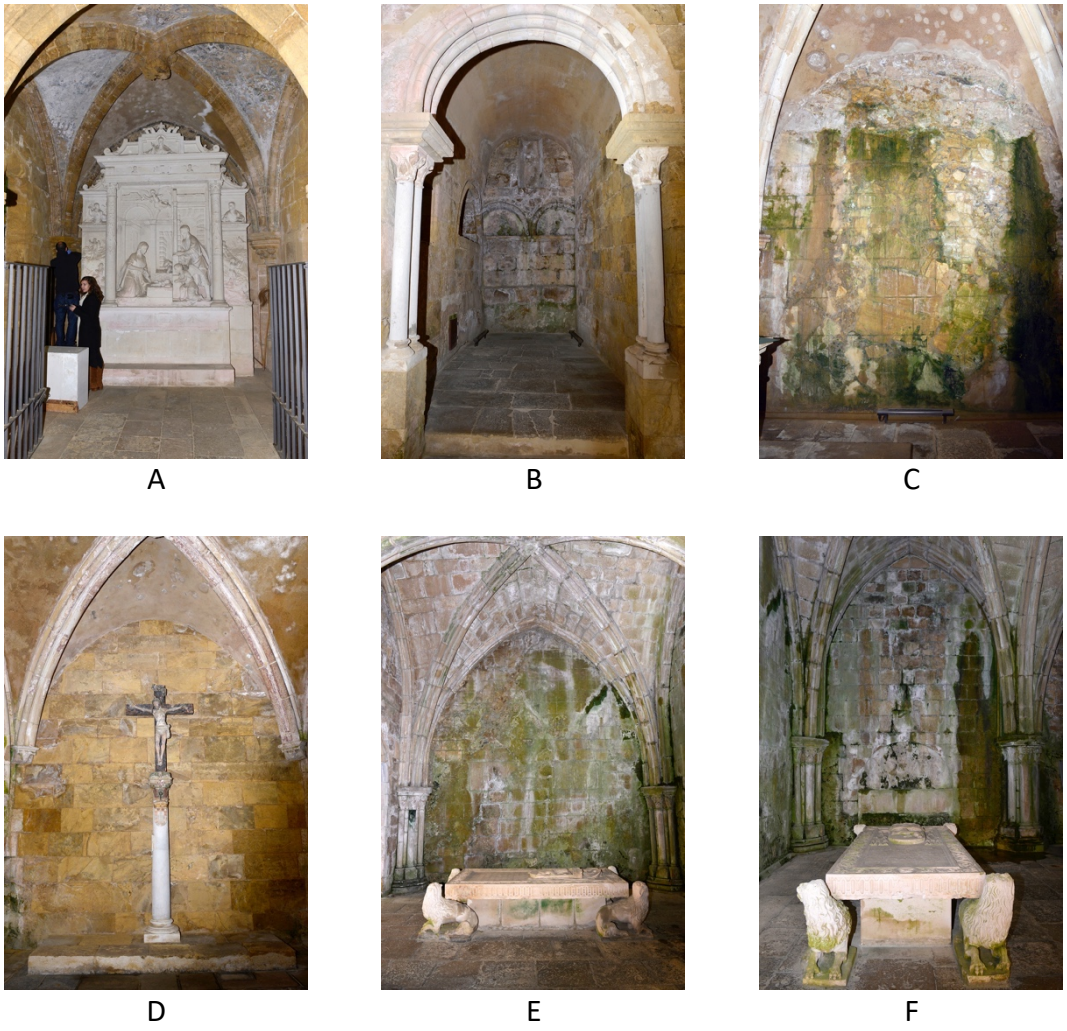

Figure 4 - The Chapels (A - Natividade; B - São Miguel; C and D - Santa Maria; E and F - São Nicolau)

Chapel of Natividade was in very good condition, and apart from the ceiling, there were very little signs of biodeterioration on the stone; Chapel of São Miguel displayed dry walls with some signs of biodeterioration and biofilm formation on the walls, some of which with dark and green biofilms with salt efflorescences; Chapel of Santa Maria presented both dry and wet walls, and a water infiltration running down one of the walls. Different signs of biodeterioration were visible, from green biofilms to black discolorations, salt efflorescences, and white patinas; Chapel of São Nicolau, displayed extensive water infiltrations, and water flowing freely through, and down the main wall. There were several places with different degrees of biodeterioration, from thick and abundant algal biofilms, to dark biofilms, dark discolorations, and salt efflorescences.

X.2.2 – Air Sampling

Air sampling was performed with a SAS Super ISO 100 bio-collector (SAS, Italy), to sample 100L of air, using an airflow of 100L/min, impacted in ⌀90mm culture plates with different media, including with 0,5g/L of streptomycin, to prevent bacterial growth.
For this purpose, three different agar media were used, to maximize the retrieval amount of species: Malt extract agar (MEA), Potato Dextrose Agar (PDA), a generalist medium used for the isolation of yeast and fungi; and Dichloran-glycerol agar (DG-18), a selective medium for low water activity and xerophilic molds isolation (Borrego and Perdomo, 2012, 2016).

These samples were collected in two different seasons (Fall 2016 and Summer 2017), in the center of each Chapel (Figures 3 and 4) and also in the central square of the cloister (Figures 2 and 3).
Culture plates were incubated at 28±1ºC for 7 days. After this time, colony counts were performed, and the different colonies were isolated into axenic cultures for further morphological and molecular analysis.

X.2.3 - Fungal Identification

The DNA extraction from obtained isolates was performed using the RED Extract-N-Amp™ (Sigma-Aldrich) DNA extraction kit, according to Trovão et al. (2019). The total ITS region was amplified by PCR, using primers ITS4 and ITS1F (White et al., 1990; Gardes & Bruns, 1996). The PCR reactions (PCR Mix: 12.5 µl of NZYTaq Green Master MIX (NZYTech™), 0.5 µl of each primer (10 mM), 10.5µl of ultra-pure water, and 1 µl of template DNA, for a final reaction volume of 25 µl) were performed on an ABI GeneAmp PCR System 9700, using the following conditions: initial denaturation at 95 ºC for 2 min, followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing at 53 ºC for 1 min, and extension at 72 ºC for 1 min, with a final extension at 72 ºC for 5 min. Visual confirmation of the overall amplification of the ITS region was performed using agarose gel electrophoresis (1.2%) stained with GreenSafe Premium (NZYTech™) and photographed in an image capture device (Bio-Rad Gel Doc XR™). Amplification products were sequenced using an ABI 3730 genetic analyzer, with the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were screened for errors and ran in NCBI's BLAST (Basic Local Alignment Search Tool) and Unite databases for similarity with published sequences. The morphological confirmation was based on the microscopic visualization using lactophenol cotton blue dye and illustrated manuals (Samson et al., 2010).

X.2.4 - Statistical Analysis

To study the diversity of fungal airborne communities, statistical analysis was performed calculating the Shannon-Wiener index ($H' = -\sum Pi \ln (Pi)$) and the Species Evenness ($E = H' / \ln (S)$), for each chapel and the exterior, and both seasons.
The Species Richness (S) is a way to quantify the number of different types (i.e., species) each dataset contains – the higher the value, the higher the number of different species in each sample – however, it does not take into account the abundance of each of the

types. The Species Evenness measures the relative abundance of the different species that make the richness in a given dataset. It is said that Diversity increases as Species Richness and Evenness increase.
The Shannon-Wiener index is therefore used to assess that diversity in categorical data. It analyses entropy, treating the species distribution and the size of a population as a probability, and is used to determine biodiversity values taking into account the Species Richness, the dominant species, and their distribution (as relative abundances) (Frosini, 2006). The Shannon-Wiener index values have to be within the range of ($0 < H < \ln(S)$) - the higher the value, the higher the statistically determined diversity.

X.3 - Results and Discussion

X.3.1 - Colony Forming Units Counts

The first step in understanding the fungal air burden is to assess the number of Colony Forming Units (CFU) present in the air - the number of propagules present in a given volume of air (usually one cubic meter) that can produce a viable colony if the proper conditions are met. In this case, it was performed by counting the number of growing colonies in the impacted plates, and then a standardized formula was applied to the count according to the manufacturer's instructions, to extrapolate for the standard volume of one cubic meter of sampled air. The values for the five sample sites, using the three different culture media, and for both seasons, are presented in Figure 5.

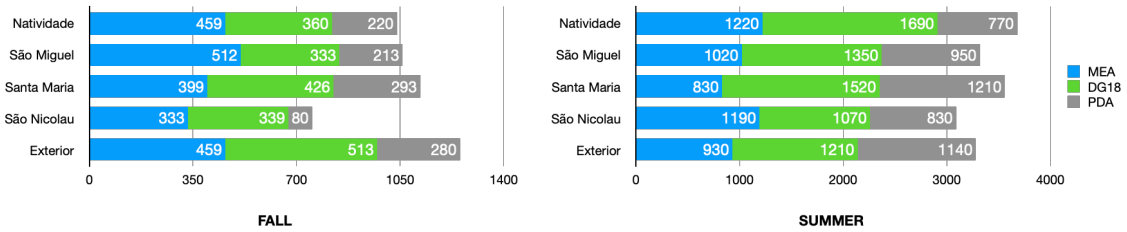

Figure 5 – Colony Forming Units count per cubic meter, in each site, season, and culture media type (MEA - Malt Extract Agar; DG18 - Dichloran-Glycerol Agar; PDA - Potato Dextrose Agar).

The number of CFU/m^3 varied between 250 CFU/m^3 (São Nicolau) and 1226 CFU/m^3 (Natividade) when considering the average number of CFUs obtained compounding the three culture media results. Using different culture media provided a higher number of species, since different organisms have different nutrient requirements and growth rates. Although spore count levels in indoor air samples are generally lower than levels in outdoor air samples, this is a different case since it is a semi-open environment, with no physical barriers between the exterior and the chapels. It is also important to assess the actual species composition – a small amount of more harmful, or potentially dangerous species, in what concerns biodeterioration potential or human health effects, can be more relevant to the study than a large amount of common, non-harmful organisms. The organism identification is addressed in Section X.3.2.

Overall, the number CFUs was much larger in the Summer season than in the Fall, which is likely due to the increased temperatures that favor sporulation in most fungal organisms, but also because of the lower humidity, which allows spores to stay airborne for longer periods, however, this did not translate into a higher number of species or diversity, as will be discussed later in this article. Within each season, the total number of CFUs was approximately the same on the different chapels, averaging between 347 CFU/m^3 in the Fall and 1128 CFU/m^3 in the Summer. Noticeably, São Nicolau showed a considerably smaller CFU count in the Summer (250 CFU/m^3), likely a consequence of the higher humidity that is characteristic of that chapel because of the almost permanent water runoff in its walls, associated with the seasonal rains.

Fungi, fungal spores, hyphae, and by-products are ubiquitous in nature, however, dampness and molds should not be allowed in environments such as homes and public buildings, since fungi in damp and water-damaged buildings play an important role in public health and disease prevention (Yang and Johanning, 2007). According to Burge and Chang (1996), and their review of quantitative standards and guidelines for fungi in indoor air, there is a very broad range of accepted values for the CFU counts in indoor air samples, and although our work was performed in a semi-open environment, visitors and staff are actually in contact with contaminated air and are therefore exposed to several potentially harmful fungi. For example, the National Health and Welfare, Canada (Malmberg, 1991) and the WHO (1988), consider that the existence of pathogenic and toxigenic species detection is unacceptable in indoor air, and both determine 500 CFU/m^3 as the upper limit for acceptable air quality if represented by more common, less harmful species. In fact, this is the same value stated on the Portuguese ordinance from 2013 (based on the Technical note NT-SCE-02). The Healthy Buildings International (Binnie, 1990), states 750 CFU/m^3 (bacteria and fungi combined) as the upper limit if species are not infective or allergenic. IAQ Association Inc. (1995), defines as a standard 300 CFU/m^3 of common fungi, and the USOSHA (1992), considers the air burden value >1000 CFU/m^3 to be contaminated air. We have detected many potentially toxigenic and potentially pathogenic species (described later in this manuscript) and obtained CFU values that often exceeded most guidelines recommendations (Rao et al., 1996), as well as the Portuguese legislation, which is an added motivation to further research potentially useful methods in the control and cleaning of the air within this monument, and others that share the same issues.

X.3.2 - Species Identification

The species that were successfully isolated into axenic cultures from the culture plates, followed the molecular identification, based on analyses of ITS rRNA gene fragments, with morphological confirmation. Considering both seasons and all locations, a total of 61 different species were obtained. 48 different in the Fall and 22 in the Summer. Many species were exclusive from a particular season (Table 1). And although there were higher CFU counts in the Summer, this didn't represent more species.

One possible explanation for this is that different environmental conditions prompt sporulation during different seasons for different species, but one cannot discard the fact that there was a much more limited amount of CFUs in the Fall, likely due to higher

humidity in the air, as a dryer air usually contains a higher spore amount (Medrela-Kuder, 2003; Awad et al., 2020).

Table 1 – Fungal species that were exclusively found in samples from one of the two seasons

Species only present in Fall		**Species only present in Summer**
Ascochyta pisi	*Neophysalospora eucalypti*	*Aspergillus flavus*
Aspergillus niveoglaucus	*Penicillium adametzioides*	*Aspergillus fumigatus*
Aspergillus ochraceus	*Penicillium aurantiogriseum*	*Aspergillus niger*
Aspergillus versicolor	*Penicillium cairnsense*	*Aspergillus tonophilus*
Beauveria bassiana	*Penicillium chrysogenum*	*Cladosporium delicatulum*
Cladosporium aphidis	*Penicillium digitatum*	*Penicillium decumbens*
Cladosporium perangustum	*Penicillium expansum*	*Penicillium* sp.
Cladosporium psychrotolerans	*Penicillium glabrum*	*Penicillium spinulosum*
Cladosporium sphaerospermum	*Penicillium simplicissimum*	*Pestalotiopsis* sp.
Colletotrichum fructivorum	*Penicillium ubiquetum*	*Phlebiopsis gigantea*
Colletotrichum siamense	*Pestalotiopsis maculiformans*	*Rhizopus stolonifer*
Colletotrichum sp.	*Phomatospora dinemasporium*	*Stemphylium vesicarium*
Cytospora eucalypticola	*Schizophillum commune*	
Diaporthe leucospermi	*Sporobolomyces roseus*	*(total of 12 exclusive species)*
Diaporthe sackstonii	*Stagonosporopsis*	
Diaporthe sp.	*cucurbitacearum*	
Discosiapseudoartocreas	*Strigula nitidula*	
Fusarium graminearum	*Talaromyces purpureogenus*	
Fusarium roseum	*Trametes versicolor*	
Leptosphaeria sp.		
	(total of 39 exclusive species)	

Overall (Figure 6), the most common species were *Penicillium brevicompactum (~9,7%), Cladosporium cladosporioides (~7,8%), Fusarium graminearum (~6,3%), Cladosporium perangustum (~5,8%), Epicoccum nigrum (~5,8%), Alternaria infectoria (~4,6%), Alternaria alternata (~4,3%)* and *Cladosporium delicatulum (~4,3%)*. An interesting observation is that the most abundant species in the chapels were also found in the samples from the central square, suggesting that the air flows between these places are likely to carry fungal propagules between them, acting as a vector for dispersion. One can assume that there are mainly three pathways for the spreading of fungal propagules to the air: one is the influx of air coming from the outside of the monument; a second one is the sporulation of existing colonies that are already present in the monument, and the last one is when different animals transport them to the inside of this monument, either insects, birds, humans, etc.

It is also interesting to note that most species (41 of 61) were only present in the air samples from within the chapels and were not present in the exterior samples. This supports the idea of the specificity and environmental requirements of most retrieved isolates and shows that the chapels have the potential to host a large set of organisms that are not present elsewhere.

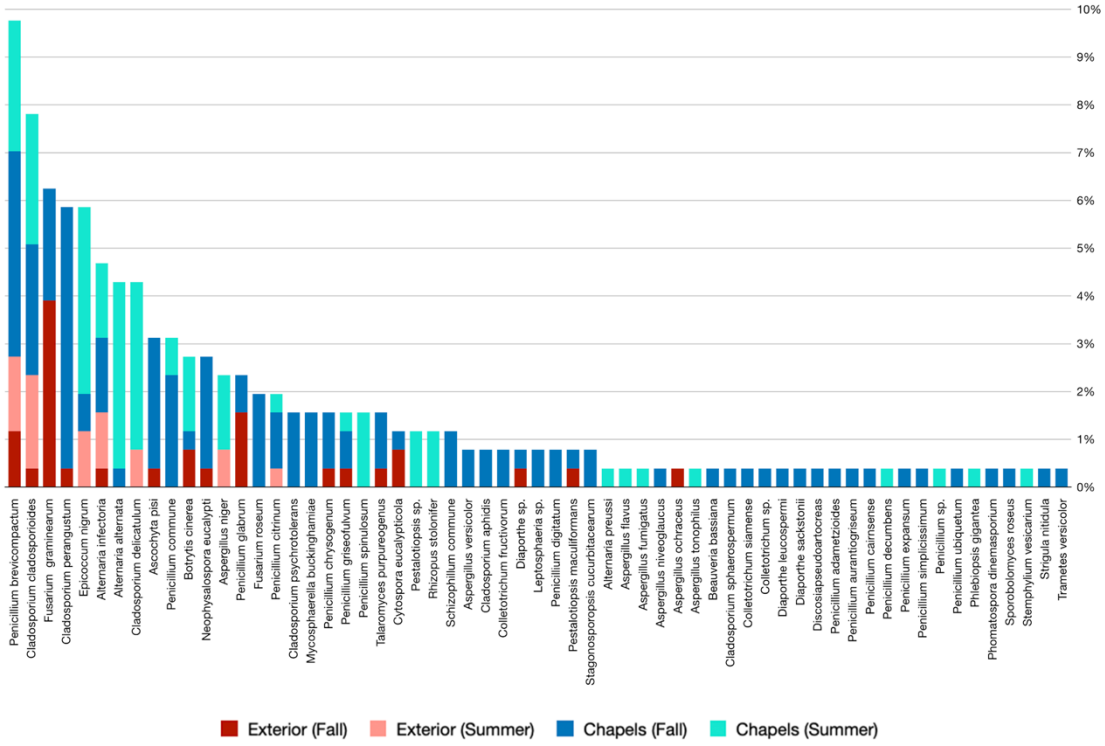

Figure 6 – Relative frequency of all species, comparing Exterior and Chapels for both seasons

The relative frequency of species in the different chapels and exterior is displayed in Figures 7 and 8 for Fall and Summer, respectively. As previously stated, many species are exclusive to just one of the sampling seasons (see Table 1). These were intentionally left blank in both charts, highlighting the differences in species composition between both seasons.

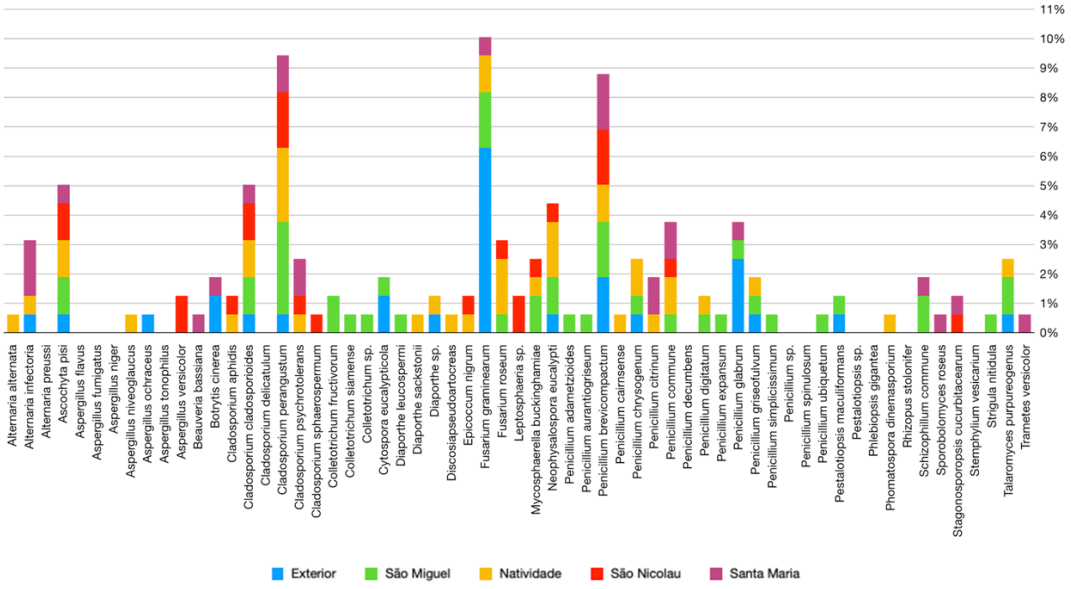

Figure 7 – Relative frequency of fungal isolates in each site (Fall 2016)

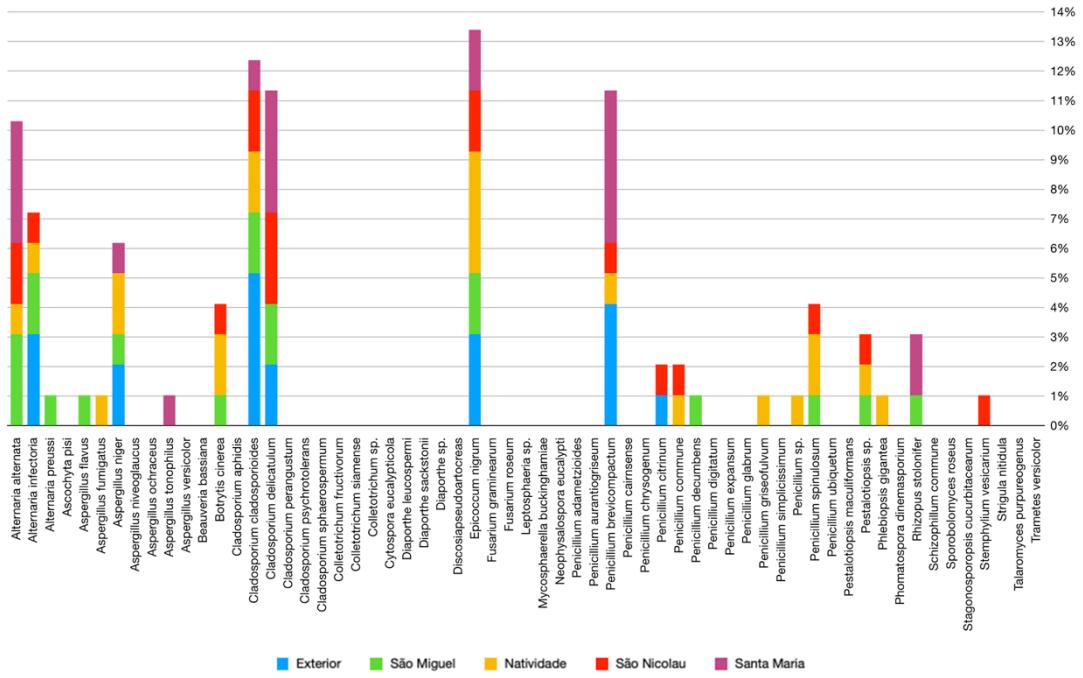

Figure 8 – Fungal isolate relative frequency in each sample site (Summer 2017)

While most frequent species were found in almost all sites, less frequent species were specific to particular chapels, like some *Aspergilli* and *Penicillia.* This may be related to the niches made available in such places that provide the adequate conditions for those species to grow and establish themselves, for being better adapted to specific environmental settings - these might be higher salinity, lack of competition, specific nutrient availability, water availability, temperature and humidity ranges, etc. This is not surprising if one considers there is an exchange (bi-directional) of propagules between the air and the surfaces), and that the presence of some propagules in the air is due to the sporulation from species growing in the walls.

Many of these organisms, as will be discussed later, are linked to biodeterioration and may have pathogenic and toxigenic effects in humans (Sections X.3.4 and X.3.5).

X.3.3 - Fungal Diversity Analysis

The statistical analysis and diversity indices (explained above) for both sampling seasons are presented in Tables 2 and 3.

According to the Shannon-Wiener Index, there was a higher diversity during the Fall in all sample sites. Since there were far more species retrieved in all five sites during the Fall than in the Summer (Species Richness, Tables 2 and 3), the Evenness values are not meaningfully different between them, even though there is a difference in the Diversity Index.

Table 2 – Statistical results and diversity indices for each site during the Fall of 2016.

Sample Site	Species Richness (S)	Shannon-Wiener Index of Diversity (H')	Species Evenness (H'/ln(S))
Natividade	25	3,1	0,96
São Miguel	27	3,2	0,96
Santa Maria	16	2,7	0,96
São Nicolau	15	2,6	0,96
Exterior	16	2,4	0,86

Table 3 – Statistical results and diversity indices for each site during the Summer of 2017.

Sample Site	Species Richness (S)	Shannon-Wiener Index of Diversity (H')	Species Evenness (H'/ln(S))
Natividade	14	2,5	0,95
São Miguel	13	2,5	0,97
Santa Maria	8	1,9	0,91
São Nicolau	12	2,4	0,96
Exterior	7	1,8	0,95

Although the number of CFU counts was much larger in air samples from the Summer, the diversity was much lower, which means that there is a lower number of different species, not only per site, but also in total. During the Fall, 48 different species were obtained (from which 39 were exclusive to this season (Table 1)), contrasting with the 22 that were isolated from the Summer samples (from which 12 were exclusive to this season (Table 1)). This is displayed in Figures 7 and 8, which show the dominance of some species. In fact, in the Summer samples, 5 species represent almost 50% of all isolates: *Epicoccum nigrum* (~13%)*, Cladosporium cladosporioides* (~12%), *Cladosporium delicatulum* (~11%), *Penicillium brevicompactum* (~11%), *and Alternaria alternata* (~10%); Whereas in the Fall samples, the 5 most frequent species represent just ~38% of all isolates: *Fusarium graminearum* (~10%), *Cladosporium perangustum* (~9%), *Penicillium brevicompactum* (~9%), *Ascochyta pisi* (~5%), *Cladosporium cladosporioides* (~5%).

X.3.4 – Pathogenic and Toxin-Producing Organisms

Concerning the health-related “air quality” of the chapels (for visitors and staff of the monument), many different potentially harmful species were identified from our air samples. From these, *Aspergillus versicolor, Aspergillus flavus, Aspergillus ochraceus, Aspergillus fumigatus* are considered toxigenic species (Portuguese ordinance from 2013, based on the Technical note NT-SCE-02). Common indoor and outdoor species from genera *Alternaria*, *Penicillium*, *Aspergillus, Epicoccum, Cladosporium*, are known producers of type I allergens, and immunoglobulin (Ig)E sensitization. They can also be sources of type III (or IgG-inducing) allergens, and can, at high concentrations, promote combined type III and IV allergic reactions, including hypersensitivity pneumonitis. (WHO, 2009, Baxi et al., 2016). Species of the genera *Alternaria* and *Aspergillus* have

been shown to produce a variety of allergens, including “Cla h I” (*Cladosporium herbarum*), “Alt a I” and “Alt a II” *(Alternaria alternata*) and “Asp f I” and Asp “f III” (*Aspergillus fumigatus*), and this becomes particularly relevant since the latter two were detected in our air samples. *Aspergillus fumigatus* and *Alternaria alternata* sensitivity in particular, but also, more broadly, other species from genera *Cladosporium*, *Penicillium*, and *Aspergillus*, have been associated with severe persistent asthma and other respiratory problems (Downs et al., 2001; Gupta et al., 2012; Knutsen et al., 2012). *Aspergillus fumigatus, A. flavus, A. nidulans,* and *A. niger*, all found in our samples, are considered opportunistic pathogens especially in the case of predisposed individuals, and the potential promoters of lung aspergillosis, keratitis, or otitis (Brakhage, 2005; Mendell et al., 2011). Another example of a hazardous species is *Aspergillus flavus,* and Aflatoxin B1 (a potent carcinogenic mycotoxin) producer (Thacker, 2004; WHO, 2009). *Alternaria alternata*, *Cladosporium cladosporioides*, and *Penicillium chrysogenum* are considered potentially pathogenic agents according to Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000, on the protection of workers from risks related to exposure to biological agents at work by the EU 2000/54/WE Directive, the Regulation of the Minister of Health in Poland (22 April 2005), the European Confederation of Medical Mycology (BSL) and the Institute of Rural Health in Lublin (IMW). *Alternaria infectoria*, *Aspergillus fumigatus*, and *Aspergillus ochraceus*, were all identified in the present study, and are also known human pathogens (Nielsen, 2003; Montanari et al., 2012; Micheluz et al., 2015; Shi et al., 2016).

X.3.5 – Biodeterioration-related Organisms

From the 61 different species that we retrieved from the air, many have been previously related to biodeterioration of stone and other construction materials (Warscheid and Braams, 2000; Gaylarde et al., 2003; Scheerer et al., 2009; Ortega-Morales et al., 2019; Liu et al., 2020)

One of the objectives of this work was to identify airborne species that had been retrieved from biodeteriorated spots from this monument (Trovão et al., 2019). By matching retrieved species from both works, we found 14 species in common, and most of these were actually found in both air and stone in the same chapel (Table 4). Some of these species were recently proven to cause biodeterioration in dolomite (the main lithotype in this site), through different processes (Trovão et al., 2020).

This suggests the possibility of the air working as a vector for the dispersion of fungal propagules to and from the walls, revealing the relevance of air flows within these environments in the expansion of fungal contamination, as well as their consequent biodeterioration on other limestone surfaces within the same monument. It would be interesting to explore the use of different approaches in similar sites, such as air filtering units, as a means to slow this propagation and try to control the spread of potentially damaging organisms. The influence of other agents in the spreading of these organisms should not be neglected, such as the water runoffs are likely a key factor in spreading different organisms, as are the insects that can also carry propagules through long distances (Trovão et al., 2013). It is important to note that many species were found

exclusively in the air, were therefore not tested for dolomite receptivity and biodeterioration by (Trovão et al., 2020), and may also reveal biodeteriorative abilities.

Table 4 – Fungal species isolated from the air (this work), and biodeteriorated spots on limestone in the different chapels (Trovão et al., 2019; * Natividade was not a part of that study).
Blue highlights the cases in which species were found in the air and stone surface of the same chapel.

Species	**Chapels**			
	São Nicolau	**Santa Maria**	**São Miguel**	**Natividade ***
Alternaria alternata	Air and Stone	Air	Air	Air
Alternaria infectoria	Air and Stone	Air	Air	Air
Aspergillus versicolor	Air and Stone	Stone	Stone	
Botrytis cinerea	Air and Stone	Air	Air	Air
Cladosporium aphidis	Air and Stone			Air
Cladosporium cladosporioides	Air and Stone	Air and Stone	Air and Stone	Air
Cladosporium psychrotolerans	Air and Stone	Air and Stone		Air
Cladosporium perangustum	Air and Stone	Air and Stone	Air	Air
Cladosporium sphaerospermum	Air and Stone			
Epicoccum nigrum	Air and Stone	Air and Stone	Air and Stone	Air
Penicillium brevicompactum	Air and Stone	Air and Stone	Air	Air
Penicillium chrysogenum	Stone	Stone	Air	Air
Penicillium glabrum	Stone	Air and Stone	Air and Stone	
Trametes versicolor	Stone	Air and Stone		

Most of the airborne species found are known to have direct and indirect interactions in the biodeterioration of limestone and stone diagenesis, mainly due to the release of different acids and chelating agents to the substrate. Biogenic organic acids produced by filamentous fungi (i.e., oxalic and citric acids), such as *Aspergillus* spp. and *Penicillium* spp., will promote the corrosion, affecting the integrity (by dissolving minerals) of different construction materials, like concrete, limestone, granite and sandstone. Furthermore, organic acids can complex or adsorb metal cations leading to the destabilization of mineral lattices (Liu et al., 2020) Many species obtained in our work are good examples of that, namely: *Alternaria alternata* (tenuazonic acid), *Botrytis cinerea* (citric, formic, fumaric, gluconic, lactic, oxalic and tartaric acids), *Penicillium brevicompactum* (citric, fumaric and gluconic acids), *P. chrysogenum*, (citric, gluconic and malonic acids), *P. glabrum* (citric, formic and fumaric acids) (Braams, 1992; Domsch et al., 1993; Gorbushina et al., 1993; Wollenzien et al., 1995; Boniek et al., 2017). These may promote the acidification and subsequent dissolution of minerals present in limestone, as has been recently verified by Ponizovskaya et al. (2019), who found *Aspergillus versicolor* and *Penicillium chrysogenum* able to actively solubilize calcite.

Oxalic acid is a very relevant organic acid, frequently produced by fungi, that assists in the solubilization of and metals (i.e., iron, aluminum, lithium, and manganese) to form

oxalate derivates, as well as the calcium carbonate within the limestone, leading to the formation of calcium oxalates, that are deemed one of the most severe biodeterioration processes in limestone-build monuments (Gadd et al., 2014). From the list of isolates retrieved from the air in this study, *Aspergillus niger*, *A. fumigatus, Botrytis cinerea, Penicillium brevicompactum, P. chrysogenum, P. citrinum, P. spinulosum*, and *Trametes versicolor* are all examples of organisms that are known producers of oxalic acid that can potentially promote the formation of calcium oxalate crystals within a limestone substrate (Gadd et al., 2014). A list of the sites from which these were retrieved from our air samples is presented in Table 5.

Table 5 – Fungal species associated with oxalic acid production* and sites from which they were isolated (*: Pinna, 1993; Dutton and Evans, 1996; Ruijter et al., 1999; Sterflinger, 2000; Jarosz-Wilkolazka and Gadd, 2003; Gadd et al., 2014;)

Species	**Chapels**				
	Natividade	**São Miguel**	**Santa Maria**	**São Nicolau**	**Exterior**
Aspergillus niger	x	x	x		x
Aspergillus fumigatus	x				
Botrytis cinerea	x	x	x	x	x
Penicillium brevicompactum	x	x	x	x	x
Penicillium chrysogenum	x	x			x
Penicillium citrinum	x		x	x	x
Penicillium spinulosum	x	x		x	
Trametes versicolor			x		

In what regards to fungal mineral diagenesis, there were other species identified in this study that have been associated with this process: *Alternaria alternata* and *Cladosporium sphaerospermum* are both known to reduce Fe(III) and Mn(IV); while *Penicillium brevicompactum* can conduct the oxidation of Fe(II) and Mn (II); and *P. chrysogenum* can conduct the adsorption of Zn, Cd, U and Th ions (Grote, 1986; Gadd, 1990; Luef et al., 1991). Furthermore, *Alternaria alternata* and *Cladosporium cladosporioides* are also able to conduct MnO and FeO mineralization in their hyphae (Burford et al., 2003) and *Alternaria infectoria* is known to contribute to limestone exfoliation and biopitting (Scrano et al., 2012).

Finally, biodeterioration can also exist in the form of aesthetic damage. Some species obtained in this work (e.g. *Aspergillus niger, A. versicolor*, *Epicoccum nigrum, Fusarium gramineum, F. roseum, Penicillium citrinum, Talaromyces purpureogenus*), are known pigment producers, have pigmented aerial mycelium and/or reproductive structures, and therefore, their growth contributes to aesthetic alterations when these get released to the substrate stone matrix (Sterflinger, 2000, 2010; Kalra et al., 2020).

X.4 – Conclusions

This study supports the relevance of the microbiota in buildings, present in the air and surfaces, as it is likely that some of the species present may present issues to the materials and humans.

Several species were retrieved from air samples from the different sites, which allowed the compassion between them, in organism abundance and diversity. The similarity of species found between chapels, and between air and biodeteriorated stone suggests that air works as a vector for the spreading of fungal propagules within these environments. The data presented in this work concerns the air bioburden of the cloister of the Old Cathedral of Coimbra. Although located outdoors, the sampled Chapels can be considered semi-closed and a characteristic bioaerosol formation is to be expected from the interaction between air and stone, as has been described above, emphasizing the specific taxa that was common to the air and biodeteriorated stone in particular chapels.

Furthermore, different biodeterioration-related and toxigenic species were found. The presence of such threats promotes the irreversible degradation of our heritage while risking not only its cultural and historical values but also the health of the working staff and visitors.

As happens in indoor environments, characterizing the potential relation between surfaces and the surrounding air may prove itself invaluable when defining preventive strategies to reduce potential negative effects caused by the rise in tourism affluence in places such as UNESCO Heritage monuments. The biological damage to cultural heritage is sometimes neglected, or not addressed appropriately, possibly due to the difficulties of implementing preventive measures.

It is our opinion that biologists, in particular those focusing on these types of microorganisms, are asked to collaborate with the teams that work in conservation and restoration, to assist (with a biological perspective and mindset) the creation of protocols and guidelines for the prevention of further biological contamination.

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