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SESSILE AEROBIC MICROBIOTA FROM THE WALL OF THE NATIONAL MUSEUM, BRAZIL: CHARACTERIZATION AND QUANTIFICATION

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ABSTRACT

The interest in preserving cultural heritage sites and artifacts has driven the development of additional measures to protect them from deterioration, which increases over time. The Garden of the Princess (Jardim da Princesa) located at the National Museum in Rio de Janeiro, Brazil, was the main site under evaluation in this work. Microbial colonization of the garden's walls was evaluated at two different areas by cultivation-based approach using specific media for fungi and bacteria, including total heterotrophic bacteria, acid and iron-producing bacteria. Results demonstrated that the higher cell density was detected in the sample from the higher humidity despite the limited humidity of the walls. Molecular identification of isolates revealed that Arthrobacter was the main bacterial genus in the biofilm, even though the others predominant genera have longer survival times during starvation and higher resistance to desiccation. Sessile fungi, despite fewer in number, were also quite diverse. In conclusion, even found in such hostile environment an interesting microbial diversity was observed in this study including four genera not yet reported as biodeterioration agents in heritage sites: bacteria *Ensifer*, *Enterobacter* and *Srenotrophomonas* and the fungus *Hypocreacea.*

Keywords: Jardim da Princesa, biodeterioration, biofilm, cultural heritage.

INTRODUCTION

The cultural heritage of a country is the legacy, including physical and intangible attributes, which make references to the identity, action and memory of different social groups. Therefore, cultural heritage includes natural environments such as landscapes, historic places and sites, buildings, and intangible items such as collections, past and continuing cultural experience, and knowledge (Tomaz, 2010; Ghirardello *et al.*, 2008; McKercher *et al.*, 2005).

One peculiarity of human civilization, since prehistory, is the ability to build temples and monuments motivated by religious principles or simply to show power (Mapelli *et al.*, 2012). Contemporary society finds in its cultural heritage their collective memory and is responsible to keep this living legacy for future generations. Therefore, the appreciation of historic monuments and the implementation of measures to maintain the cultural heritage of each nation is important (Tavin, 2004).

The preservation of assets exposed to outdoor environments is complex. Under this condition, the action of different physical and chemical factors such as rain, salinity, wind and air pollution, in association with microorganisms activity, particularly increases the wear of the materials, which contributes to the environmental disfigurement and restoration costs (Loh, 2011; Shirakawa *et al.*, 2008; Gaylarde and Gaylarde, 2005). The type of material and coatings used in buildings also has an influence on the colonization and biodeterioration. After metallic materials, concrete is the most susceptible material to deterioration process, which can be seen on its surface through crusting, loss of material, and color alterations (Resende, 2008; Sarró *et al.*, 2006). In addition, in outer walls, the composition of the structural material and the paint and dirt that accumulate in them can also be a nutritional source for microbial growth (Ciferri, 1999).

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The presence of microorganisms can increase the deterioration process by accelerating the kinetics of the reaction without altering the electrochemical phenomenon. However, the intensity of the biodeterioration depends on the amplitude of the microbial colonization, their composition, and their distribution on the surface (Beech and Gaylarde, 1999). In addition to the aesthetic problems, microbial colonization of solid surfaces can compromise the structure of the material and also be a health risk since bacterial and fungal spores can trigger respiratory problems and allergic reactions. Therefore, the biodeterioration process has been extensively studied since it involves constructions and human housing, such as bridges and buildings, and also cultural legacy represented by sculptures, statues and monuments (Sanchez-Moral *et al.*, 2005; Mapelli *et al.*, 2012).

The microbial colonization involves the adhesion of microorganisms to a given structure and the formation of biofilms. Biofilms are complex structures consisting of different microbial species embedded in a matrix made of extracellular polymeric substances (EPS), basically polysaccharides, and high content of water. In the biofilm, the coexistence of microorganisms of different metabolisms, and even the death of the cells enables a continuous supply of nutrients, which ensures the stability of the structure (Herrera and Videla, 2004; Costerton and Wilson, 2004).

In walls, different species from different microbial groups have been isolated, including bacteria, fungi and algae (Wei *et al.*, 2013; Murphy, 2002; Flores *et al.*, 1997). Among them, there are heterotrophic bacteria, particularly EPS-producing, considered primary colonizers, since the EPS favor the adhesion of cells to surfaces and its presence in biofilms allows the adherence of new microorganisms, and may serve as a substrate. The biogenesis of organic or inorganic acids may weaken the mineral structure of the wall or of the coating applied to them and also cause color changing. These acids may also encourage the development of other microbial species. The recurring problems of iron bacteria activity in outdoor walls are changes of color and the support for sulfur oxidizing bacteria activity. Fungi are mainly involved in the release of metabolites, which are often corrosive, whereas the mycelium contributes to the structure of the biofilm. However conventional microbiological techniques usually employed to characterize the microbial communities involved in biodeterioration processes allow only the identification of a small portion of the microorganisms involved (Lan *et al.*, 2010; Gu *et al.*, 1998).

The objective of this study was to investigate the microbial diversity on walls surrounding the Garden of the Princess, created by D. João VI in 1818, that was part of the residence of the Portuguese imperial family. Presently, the building is part of the National Museum of Rio de Janeiro, the largest museum of natural and anthropological history of Latin America. This is the first study characterizing biofilms on this site. The microbial characterization will allow better assessment of possible risks to property, as well as further insight into the conservation methods to be adopted (Lopez *et al.*, 2010).

MATERIALS AND METHODS

Site and Sampling Procedures

The Garden of the Princess is located in the Quinta da Boa Vista Park in the city of Rio de Janeiro, an urban area but with little vehicle traffic, wet and wooded. Samples were collected at two different areas in the concrete walls coated with paint. The wall is in a state of deterioration since the last recovery occurred more than 30 years ago (Fig. 1). The main constituents of the concrete, determined by X-ray diffraction were as follows: SiO_2 (60.6%), CaO (18.2%) and Al_2O_3 (4.0%).

The samples were collected by scraping the wall with a sterile spatula and a flexible plastic mold, disinfected by immersion in alcohol, to restrict the scraping zone to a known area. The mold had a square inner opening with $4cm^2$. Ten samples from each spot were collected for the microbiological analyzes. The samples were collected in penicillin bottles with 50 ml capacity containing 40 ml of reducing solution (0,85g/L sodium chloride, 0,134 g/L sodium thioglycolate, 0,1g/L ascorbic acid) previously weighed on an analytical balance to the tenth of a milligram. Samples were also collected in sterile Petri dishes to determine the moisture content of each material collected. Seasonal temperature in the area where the National Museum is located varied from 15.1 to 28.0°C, the relative humidity was between 31 to $81g/m^3$ and the average rainfall was 5.5 mL/day [data obtained during 15 days before sapling].

Microbiological Analyzes

The collected samples, previously homogenized and diluted, were poured on Petri dish using the spread plate technique. Different specific culture media were used including media for heterotrophic bacteria, acid producing bacteria and iron bacteria, as well as for fungi (Table 1), with addition of antimicrobials (nystatin or amoxilicin, $50mgL^{-1}$) to inhibit undesirable contaminants.

The specific media for growth of heterotrophic bacteria, acid producing bacteria, iron bacteria, and fungi were incubated in bacteriological incubator at 30°C for 2, 3, 4, and 5 days, respectively. After the incubation time the colonies were counted (colony forming units, CFU), or the growth was evaluated through the most probable number technique (MNP). All analyzes were performed in triplicate and the results shown in CFU/ cm^2 or NMP/cm^2.

Fig. 1. Wall from the garden of the princess. Numbers represented sampling sites.

Table 1. Cultivations conditions employed to determine the diversity of biofilms formed on the walls of the Garden of the Princess.

Microorganisms	Counting method	Media
Heterotrophic bacteria	SP	Nutrient agar added with Nys
Acid producing bacteria	MPN	Phenol red broth added with Nys
Iron bacteria	SP	Ammonium ferric citrate agar added with Nys
Total fungi	SP	Sabouraud agar added with Amx

SP – Spread Plate counting; MPN – Most Probable Number; Nys – nystatin (50mg.L^{-1}); Amx– amoxicilin (50mg.L^{-1}).

Identification of Predominant Groups of Bacteria and Fungi

The microorganisms were recovered from predominant colonies grown on agar plates. The DNA extraction was carried by thermal lysis. One isolated colony was picked and suspended in 50 µL ultrapure water (MilliQ^{TM}) in *eppendorf* tube (500 µL). The microbial suspension was treated by heat shock using dry bath, according to the program: 96°C for 10 minutes followed by 4°C for 30 minutes. Then, the material was centrifuged for 1 minute at 10,000g. The supernatant containing the DNA was transferred to a new tube and stored at -20°C until the amplification of DNA. The DNA amplification was conducted using the conventional PCR technique (PCR System 9700 thermocycler, Applied Biosystems) using the random primers, Sadir (5'-AGAGTTTGATCATGGCTCAGA-3') and S17 (5'-GTTACCTTGTTACGACTT-3') for bacteria. For fungi, the genomic DNA of each isolate was extracted employing the commercial *kit MOBio Ultra Clean^{TM}Microbial DNA Isolation*.

After the extraction, the concentration and degree of purity of the DNA were analyzed by spectrophotometer (NanoDrop Spectrophotometer - model ND-1000) at wavelengths of 230, 260 and 280 nm. The DNA amplification was conducted using the conventional PCR technique (PCR System 9700 thermocycler, Applied Biosystems) using the random primers, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The conditions of amplification were 94°C/30 sec, 55°C/30 sec, 72°C/30 sec for bacteria and 94°C/30 sec, 50°C/30 sec, 72°C/30 sec for fungi with 30 cycles for both. All the

amplified products were analyzed in agarose gel (1%, m/v). After checking the efficiency of PCR procedures by electrophoresis, the amplified products were purified using the system Wizard® PCR Clean-Up System (Promega) according to the manufacturer's guidelines. The material was placed in a 96-well microplate containing from 30 to 60 ng of amplicon plus 2.5 pmol of primer suspended in ultrapure water (Milli-Q) qsp 6 uL. Each tube contained a single primer (Sadir and S17, for bacteria, and ITS4 and ITS5 for fungi) in duplicates per sample. The amplicons were labeled using primers in solution and 0.5 μL BigDye Terminator v3.1 reagent Standard Cycle Sequencing Kit (Applied Biosystems) in final volume of 10 μL. The labeling reactions were performed in a thermocycler LGC XP Cycler. The PCR conditions were 96°C/10 sec, 55°/5 sec, 60°C/4 sec, 25 cycles.

After the samples were purified by precipitation with isopropanol and washing with 75% to 60% ethanol, a dilution was performed with 10 μL Hi-Fi formamide (Applied Biosystems), denatured at 95°C for 5 min, cooled on ice for 5 min and electroinjecting Automatic Sequencer AB 3500 Genetic Analyzer with reinforced capillaries of 50 cm and polymer POP7 (Applied Biosystems). The sequences were edited and analyzed with an appropriate software (Chromas Lite® and Bioedit), GenBank (www.ncbi.nlm.nih.gov) and compared for similarity to other sequences already deposited by using BLAST software online.

RESULTS AND DISCUSSION

Even though the places where the two samples were collected were exposed to similar ranges of UV radiation and other climatic variations and were only 5-10 m apart from each other, both places had well-differentiated visual appearance of color and texture, revealing advanced process of biodeterioration (Fig. 1). At the time of sampling, the tropical climate characterized by mild temperatures and low precipitation, may have been the main factor for the low water content of the samples, 4.6 and 1.1%, in area 1 and 2, respectively.

The low humidity did not restrict the development of microbial biofilms on the walls. As shown in Table 2, both sampling areas had significant numbers of sessile microorganisms of different microbial groups. The highest microbial density was found in area 1, where the humidity was higher. The number of sessile microorganisms was different according to the place where the sample was taken from, particularly for iron bacteria. However, the number of fungi has remained in the same order of magnitude in both areas. The variation of microbial density may be due to variations in humidity, being lower in area 2.

Another study, Shirakawa *et al*. (2011) evaluated the characteristics of concrete walls coated with different formulations of acrylic paints for seven years of exposure to the elements, located in the urban area of São Paulo and in the coastal town of Ubatuba, State of São Paulo, Brazil. The discoloration and detachment of the coatings were more intense in Ubatuba. Phototrophic microorganisms and fungi were detected in all biofilms using standard microbiological methods. However, biofilms from Ubatuba had fewer numbers of fungi and higher number of phototrophic microorganisms. The authors attributed this fact to the higher humidity in the coastal region, due to increased precipitation.

Similarly, Guiamet *et al.* (2011), investigating the biofouling and biodeterioration of photos and maps stored at Historical Archive of the Museum of La Plata, Argentina, and two repositories of the National Archive of Cuba Republic, found an increased number of bacteria (2,148.7 CFU/m^3) and fungi (260.7 CFU/m^3) in areas where the relative humidity of the air and temperature were 75 g/m^3 and 28°C, respectively. In contrast, under 59 g/m^3 and 23.8°C, the microbial density decreased by 3 times for fungi and by 35 times for bacteria. Also, Papida *et. al.* (2000) observed that the microbial density was higher when the water content was also higher in constructions of dolomite (8.5%) and limestone (12.1%). According to these authors, the humidity increased the porosity of the material, resulting in the reduction of its strength and consequently increasing its vulnerability, leading to the erosion process.

Table 2. Biomass of cultivable microorganisms on the walls of the Garden of the Princess.

Microbial groups	**Cell counting**	
	Area 1	**Area 2**
Aerobic bacteria (CFU/cm^2)	$3.3x10^6$	$2.6x10^4$
Acid producing bacteria (MPN/cm^2)	$8.2x10^5$	$2.8x10^4$
Iron bacteria (CFU/cm^2)	$1.4x10^5$	ND
Fungi (CFU/cm^2)	$6.8x10^3$	$2.4x10^3$

ND – not detected (below the detection limit of the method).

The predominant cultivable sessile microbial cultures of each group, corresponding to 15 bacteria and 10 fungi. Table 3 shows the diversity of the microbial community in the biofilms formed on the walls of the Garden of the Princess in two different areas. According to López-Miras *et al.* (2014), bacteria and fungi that may survive as spores represent an important part of the microbial community which can accumulate on the surface for a long time.

The greater diversity of bacteria was found in the area with the lowest moisture content (area 2), which showed also the lower cell density. Likewise, in area 2 the diversity of fungi was slightly higher than that found in area 1, although the species constituents of each biofilm were quite different. Among the bacterial isolates obtained using culture-dependent methods, the *Artrobacter* genus stood out. And surprisingly, the other four genera of bacteria identified in this study have not yet been described in the literature as members of microbial communities in biofilms formed in buildings and historical heritage: *Ensifer*, *Stenotrophomonas rhizophila*, *Enterobacter* and *Rhodococcus*.

Artrobacter, belonging to the phylum Actinobacteria, is a common airborne and spore-forming microorganism inhabitant of the soil environment, in which it corresponds to more than one half of the total bacterial populations (Cross *et al.*, 2015; Scheublin and Leveau, 2013; Mongodin *et al.*, 2006). This genus, as well as *Pseudomonas* and *Bacillus*, are related to biofilm formation since they are typically heavily-encapsulated and fast-growing bacteria (López-Miras *et al.*, 2013). Interesting properties of these bacteria are the metabolic versatility, capable to growth at the expense of a minimum of nutrients and to degrade a wide variety of organic substances, including toxic ones, which contributes to recycle nutrients in the soil (Weiser *et al.*, 2014). In view of the above, the presence of these genera, in particular the genus *Arthrobacter*, in biofilms formed on the walls of the Garden of the Princess is mainly the result of the proximity of the sampling areas with soil and vegetation, as seen in figure 1. Heyrman *et al.* (2005) also isolated 21 bacterial strains from samples of biofilms from Servilia tomb (necropolis of Carmona, Spain) and the Saint-Catherine chapel (castle at Herberstein, Austria). All the isolates were allocated to the genus *Arthrobacter*, including six novel species, which proposed names were *A. castelli* spp. nov., *A. monumenti* spp. nov., *A. parietis* spp. nov., *A. pigmenti* spp. nov., *A. tecti* spp. nov. and A. *tumbae* spp. nov.

Similarly, to this present work, bacteria and fungi were isolated from microbial biofilms developing on the surfaces and within the painting layers of St Martin Church, Germany (Gorbushina *et al.*, 2004). The predominant bacteria belonged to the genera *Arthrobacter* and *Bacillus*. However, as reported by the authors, the microbial community was inactive. However, studies by Sarró *et al.* (2006) on the statues of lions, at the Alhambra Palace, Spain, attributed part of the responsibility on the monument deterioration process to *Arthrobacter*. While, Nuhoglu *et al.* (2006) stated that *Bacillus* sp. was the main responsible to the acceleration of the biodeterioration process of stone monuments in Turkey.

Among fungi, the predominance of *Fusarium* in area 1 was interesting as previous studies only identified it in isolated areas while *Cladosporium* and *Penicillium* stood out as the genera with higher prevalence (Elissawy *et al.*, 2015; Guiamet *et al.*, 2011; Nuhoglu *et al.*, 2006; Shirakawa *et al.*, 2002). Most *Fusarium* species is widely distributed in soil from temperate and subtropical regions. These fungi are able to grow in water and soil through the assimilation of organic matter in decomposition, and can also infect plants and animals. Some species are producers of mycotoxins, which can enter the food chain and affect the health of people and animals (Priest and Campbell, 1996).

In area 2, species of the *Pestalotiopsis* genus were predominant. In fact, *Pestalotiopsis* species are largely distributed worldwide (Jeewon *et al.*, 2003; Wang *et al.*, 2005). Similarly, to most fungi, they can grow from a wide range of substrata, as saprobes in soil, and plant pathogens, even though endophytic *Pestalotiopsis*are considered being the most important community in nature, inhabiting leaves, branches, stalks and seeds. Some endophytic *Pestalotiopsis* have been shown to produce important bioactive products such as antimicrobial and antitumor compounds of potential application in medicinal area (Wei *et al.*, 2007; Li *et al.*, 2001). Endophytic *Pestalotiopsis* have been isolated mainly in the subtropical and tropical regions (Liu *et al.*, 2007). In Brazil, *Pestalotiopsis* species have been reported in almost all the territory, although its identification is difficult due to the known morphological variation between different species (Jeewon *et al.*, 2003). Ramos-Mariano *et al.* (1998) found *P. palmarum* to be the main fungus in coconut in Brazil. More recently, two fungal species, *P. neglecta* and *P. maculiformans*, were isolated from the air inside the imperial coach, first used in the consecration and coronation ceremony of King Pedro II in 1841, and then on solemn occasions (Lutterbach *et al.*, 2013). Currently, the coach is part of the Imperial Museum collection, located in Petropolis, Rio de Janeiro.

Gorboushina *et al.* (2004) isolated thirty-two fungal species by cultivation methods. Most of the fungi were identified as *Acremonium*, *Aspergillus*, *Cladosporium*, and *Fusarium*. A *Cladosporium* specie was also detected in one of the biofilms of this present work, but unlike no *Aspergillus* species and only one *Pestalotiopsis* specie

Table 3. Microbial diversity of the biofilms on the walls of the Garden of the Princess.

Area 1		**Area 2**	
Bacteria	S	Bacteria	S
Artrobacter spp.[1]	85%	*Artrobacter* sp[1]	99%
Artrobacter sp.[1]	100%	*Bacillus megaterium*[3]	100%
Artrobacter spp.[1]	100%	*Bacillus megaterium*[3]	100%
Artrobacter spp.[1]	100%	*Enterobacter* sp.[4]	84%
Artrobacter spp.[1]	100%	*Enterobacter* sp.[4]	91%
Ensifer spp.[2]	97%	*Rhodococcus* sp.	90%
Ensifer spp.[2]	92%	*Stenotrophomonas rhizophila*	100%
Pseudomonas mexicana	100%		
Fungi	S	Fungi	S
Hypocreaceae sp.	100%	*Cladosporium* spp.	100%
Fusarium oxysporum	99%	*Hypocreales* spp.	100%
Fusarium domesticum	96%	*Pestalotiopsis* spp.[5]	99%
Penicillium spp.	99%	*Pestalotiopsis disseminata* [5]	99%
		Pleosporales spp.[5]	99%

S – similarity. [1] Six different isolates *Artrobacter*. [2] Two different isolates *Ensifer*. [3] Two different isolates *Bacillus megaterium.* [4] Two different isolates *Enterobacter*. [5] Three different isolates *Pestalotiopsis.*

could be detected by the conventional cultivation technique. This is unusual since spores of those fungi are frequently present in the air and soil. However, the investigation of microbial communities adhered to the obverse and the reverse sides of an oil painting on canvas, exhibiting signs of biodeterioration, employing culture-dependent technique also revealed the presence of solely *Penicillium* spp. (López-Miras *et al.*, 2013). According to the authors, the results may be related to the indoor quality of the air of the room where the painting was exposed. Air samples showed bacteria and fungi numbers ranging between 200 and 500 CFU/m^3, including fungal isolates from the genera *Alternaria*, *Ulocladium*, *Stachybotrys*, *Cladosporium*, and mainly *Penicillium* (46.2 % phylogenetically identified). *Mucor*, *Aspergillus* and *Eurotium* were solely detected through genetic techniques. Besides, differences in the community structure could be observed when using different techniques, wherein in some cases the counts were below the detection limit of the technique used.

In this study, the *Hypocreales* fungus was one of the most predominant in the biofilms. This is the first report on their presence associated to communities adhering to solid surfaces. These microorganisms form haustoria and occur naturally as parasites of plants and have a vibrant color in the fruiting body, usually yellow, orange or red. Species of this genus are also found in the lichen form (Silanes *et al.*, 2009). There are reports of the isolation of *Hypocreales* spp. from the anaerobic fermentation of organic waste (Kazda *et al.*, 2014) and marine-derived *Hypocreales* capable of producing bioactive terpenes (Elissawy *et al.*, 2015).

The present study demonstrates that structures of the same area are subject to different microbial colonization, which is a critical problem when setting the restoration strategy of cultural heritage. Moreover, it was possible to identify the presence of fungi and, mostly bacteria, whose metabolism can aggravate the deterioration by releasing substances that promote the change in color of the walls, or whose corrosive action damages the structure and coatings usually used for the protection of these buildings. Most of the identified species have low nutritional requirements and are high enzymatic producers of extracellular enzymes, such as proteases, esterases and lipases, which activities contribute to the loss of material (Ciferri, 1999). As pointed out by some researchers, the correct conservation and restoration of biodeteriorated buildings and monuments needs a detailed knowledge of the microbial communities associated with these substrates and changes of the natural environment and climate conditions (Capodicasa *et al.*, 2010; Suihko *et al.*, 2007; Schabereiter-Gurtner *et al.*, 2001). This type of research is still little explored in Brazil which has allowed many of the artifacts and monuments of the Brazilian heritage suffer from progressive deterioration. Therefore, ways to control biofilm formation must be studied in order not to miss the cultural heritage left by our ancestors.

CONCLUSION

The analysis of the microbial community adhered to the outdoor walls of the Garden of the Princess, part of the National Museum of Rio de Janeiro, Brazil was performed using culture-dependent techniques. Our

results demonstrated that fungi and particularly bacteria were the main members of the microbial communities adhered to the surfaces, despite the limited humidity of the walls. Molecular identification of isolates revealed that *Arthrobacter* was the main bacterial genus in the biofilm, even though the others predominant genera have longer survival times during starvation and higher resistance to desiccation. Sessile fungi, despite fewer in number, were also quite diverse in the communities' structures. Our results confirm that bacteria of the genera *Arthrobacter*, *Bacillus*, *Pseudomonas* and fungi of the genera *Pestalotiopsis*, *Pleosporales*, *Fusarium*, *Penicillium* and *Cladosporium* are widespread in microbial communities adhered to outdoor walls in tropical areas. In addition, three bacterial species that were identified as *Ensifer* spp., *Stenotrophomonas rhizophila*, *Enterobacter* spp. and one fungi species, *Hypocreaceae* spp., have not yet been described in wall as biodeterioring agent.

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