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Role of Exposure on the Microbial Consortiums on Historical Rural Granite Buildings

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Abstract: Local granite has been used throughout history in Galicia (NW Spain), forming the basis of much of the region's architecture. Like any other rock, granite provides an ecological niche for a multitude of organisms that form biofilms that can affect the physical integrity of the stone. In this study, for the first time, characterization of the microbial consortium forming biofilms that developed on historical rural granite buildings is carried out using a combination of culture-dependent and next generation sequencing (NGS) techniques. Results pointed to differences in biofilm composition on the studied rural granite buildings and that of previously analyzed urban granite buildings, especially in terms of abundance of cyanobacteria and lichenized fungi. Exposure was corroborated as an important factor, controlling both the diversity and abundance of microorganisms on walls, with environmental factors associated with a northern orientation favoring a higher diversity of fungi and green algae, and environmental factors associated with the west orientation determining the abundance of lichenized fungi. The orientation also affected the distribution of green algae, with one of the two most abundant species, *Trentepohlia cf. umbrina*, colonizing north-facing walls, while the other, *Desmococcus olivaceus*, predominated on west-facing walls.

Keywords: biofilm; microbial community; algae; cyanobacteria; fungi; bacteria; next generation sequencing; granite; cultural heritage; Trentepohlia

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1. Introduction

Granite is one of the most commonly used types of stone in construction worldwide and is known to have been in use since Egyptian times [1]. This igneous rock is widely used in construction because of its sustainability and durability, being a hard rock that is resistant to both heat and ultraviolet radiation. Nonetheless, all materials exposed to the environment gradually deteriorate as a result of physical, chemical, and biological processes [2,3]. Interactions between the atmosphere and stone invariably lead to the formation of altered surface layers, which eventually results in damage to the original stone. This damage is of great concern in the case of stones in unique buildings and monuments [4]. Information about the biodeterioration of historical granite buildings is very limited relative to what is known about buildings constructed with limestone or sandstone [5], but it is known that the main factors leading to the granite biodeterioration process are the presence of soluble salts [6] and biodeterioration caused by colonizing organisms [7–9].

Microbial colonization alters the stone substrate causing physical–chemical and aesthetic damage. These negative effects have been widely studied [10–15]. However, the idea of the protective effect of organisms on stone has recently gained relevance as their function as consolidants, binding particles, and protecting stone from erosion or aggressive substances, as well as from other abiotic factors, has been demonstrated [16–18]. Both deteriorative and protective actions can be caused by a single species, but can also be due

to the combined, inter-related effects of various different microorganisms, which also depends on the environmental conditions [19–21]. It is therefore important to understand the complexity, composition, and structure of the community inhabiting the stone of cultural heritage buildings, in order to foresee the risk deterioration degree and, thus, be able to carry out the most appropriate conservation tasks.

Due to the interactions between organisms and substrate, with proved positive and negative effects, the development of techniques allowing the study and identification of microbial communities has gained importance as a key issue in the heritage conservation field. In this sense, different techniques have been used over time to identify organisms forming biofilms. Traditionally, their isolation, identification, and study have been carried out by means of classical culture techniques. These culture-dependent techniques have the advantage of being able to perform biochemical and physiological studies in parallel to identification; however, they greatly underestimate existing diversity [22] and favor the rapid growth of opportunistic species, making it difficult to identify other microorganisms that may play an important role [23]. With the emergence of metagenomics and, in particular, next generation sequencing (NGS) techniques, detection capacity has increased exponentially, thus improving the potential and level of detail of microbial ecology studies [24]. NGS techniques enable large amounts of data to be obtained more quickly than with any of the previous methods. Thus, the combination of these modern techniques together with traditional culture-dependent methods would enable the production of more data and a better understanding of specific findings [25]. Numerous studies on the microbial colonization of historical monuments have been carried out using these modern techniques, especially on sandstone, marble, and limestone substrates. In the case of historical granite buildings, studies characterizing microbial communities are scarce [12,26–28] and most of them have been carried out using traditional methods [29–31], with only one using NGS techniques [26]. In this study, biofilms (algae, cyanobacteria, and fungi) on historical granite buildings in Santiago de Compostela were characterized; however, bacterial communities, which are important at early stages of biocolonization, were not studied.

In this study, we investigated the microbiological colonization of three granite-built churches located in rural areas of Galicia, in the northwestern part of the Iberian Peninsula. Colonization was characterized using a combination of traditional and NGS methods (culture dependent methods for phototrophic organisms and NGS methods for fungi and bacterial organisms). The biodiversity was determined using the Shannon diversity index. Moreover, taking into account that the orientation of buildings is one of the factors that most strongly influences the development and composition of subaerial algae biofilms, as it determines the amount of light and the availability of water on the substrate [32,33], the role of exposure was also considered.

2. Materials and Methods

The three churches under study, Sta. María de Vilar (SMV) (43°8′1.2″ N, 7°54′30.1″ W; 466 m), San Cibrao de Barreiro (BAR) (42°48′25.4″ N, 7°55′20.6″ W; 509 m), and San Cosme de Rocha (ROC) (43°02′30.7″ N, 7°50′59.7″ W; 532 m) were selected as typical examples of granite constructions in rural Galicia (in the NW of the Iberian Peninsula). The churches (hereafter referred to as SMV, BAR and ROC churches) are located in rural areas, far from major cities, with low levels of pollution (Figure 1). The churches are located along a 35 km transect, from north to south, and each church is surrounded by a small graveyard and local vegetation. The region has an Atlantic climate, with a mean relative humidity of 80% and a mean yearly rainfall of 1180 mm. The mean number of days per month with rainfall is 13 and the mean temperature is 12 °C. The wind rose (the dominant frequencies of wind direction) showed clear northeastern and southwestern wind dominances (https://www.meteogalicia.gal/; Accessed: 21 January 2021).

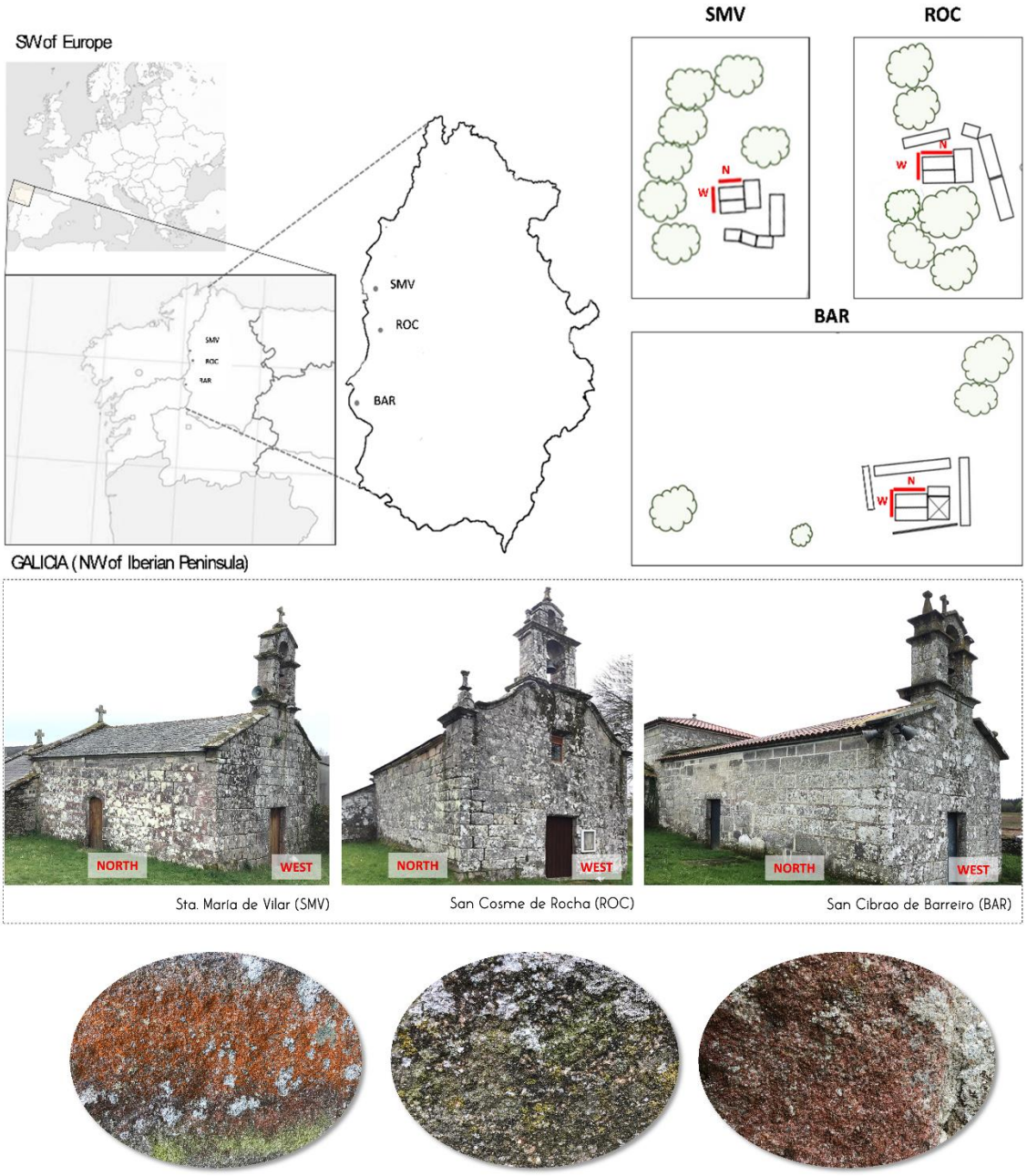

Figure 1. Location and photographs of the north and west façades of the SMV, ROC and BAR churches (Galicia, in the NW of the Iberian Peninsula) and the different biopatinas.

The church walls are almost completely covered by lichens and biofilms are only present on the north- and west-facing walls. Representative samples of the subaerial biofilms were obtained by scraping approximately 5 mg from three areas of the north (N)- and west (W)-facing walls of each church.

Phototrophs (i.e., algae and cyanobacteria) were studied by means of conventional morphological examination under light microscopy, since it allows for a reliable identification and for the estimation of the abundance of the different taxa. Bacteria and fungi were studied using next generation sequencing, since growing different taxonomic groups to obtain representative data requires great effort, and not all taxa grow in culture.

2.1. Cyanobacteria and Algae Morphological Identification

A subsample of 0.5 mg of each direct scraping was disaggregated and resuspended in 1 mL of BG11 culture medium for microscopic examination of the algal and cyanobacterial communities present on the N- and W-facing walls of each church. The subaerial biofilm species were examined under an optical microscope (Nikon Eclipse E600 equipped with an E-Plan 40x objective, N.A. 0.65), with differential interference optical contrast (Nomarski). Photomicrographs were taken with a digital camera (AxioCam ICc5 Zeiss). The algal communities were characterized using a semi-quantitative approach in which 5 aliquots of 150 µL of each sample, resuspended in medium, were examined by counting a total of 100 fields (40× magnification) and a minimum of 1000 cells. The percentages of each species present were calculated from the total number of cells observed; the species detected by culture were assigned 1% as a record of their presence in the total count. Agglomerative hierarchical analysis of the percentages of each species/taxon was carried out, and Ward's criterion was applied. The different samples of subaerial biofilm were compared in relation to the affinities between the communities by means of a Euclidean distance matrix of the dissimilarity coefficient, following the community classification criteria of Gauch and Whittaker [34].

BG11 media [35], with and without nitrogen ($BG11_0$), were prepared to promote growth of cyanobacteria (Cyanoprokaryota). Specifically, the $BG11_0$ medium was used to promote nitrogen-fixing cyanobacteria and their dormant stages. BBM medium [36,37] was used to promote the growth of green algae and the different phases of their life cycle in order to confirm their taxonomic identification. Cultures were maintained in sterile controlled laboratory conditions at a constant temperature of 23 °C, a 12:12 h light/dark photoperiod and a photon irradiance of 20 µmol m^{-2} s^{-1}. Cultures were maintained for 6 months and examined every 2 months to confirm taxonomic identifications based on morphometric characteristics and life history stages not presented in the initial sample.

Species identification and nomenclature were mainly based on Rifón et al. [31,38]. The following articles were also consulted: Škaloud [39], for green algae (Chlorophyta); Komárek [40], for cyanobacteria (Cyanophyta/Cyanoprokariota); and Lange-Bertalot and Hoffman [41] for diatoms (Bacillariophyta).

2.2. Bacteria and Fungi Next Generation Sequencing Analysis

2.2.1. DNA Extraction and PCR Amplification.

Total genomic DNA was isolated from 0.25 g of each individual biofilm sample with the QIAamp® Power® Fecal DNA Kit (Qiagen Sciences Inc., Germantown, MD, USA) following the manufacturer's protocol. DNA was quantified and quality-checked using a Nanodrop spectrophotometer. A 5-ng aliquot of total genomic DNA was used as a template for the specific PCR amplification of hypervariable V3-V4 regions of the 16S rRNA bacterial gene and of the ITS II region between the 5.8S rDNA and LSU of fungal ribosomal DNA in order to detect respectively bacterial and fungal species in the biofilm samples. PCR amplification of V3–V4 hypervariable regions in the 16S rDNA gene consisted of 25 cycles with 55 °C as annealing temperature, as described by [42]. PCR amplification conditions for the ITS region consisted of 40 cycles with 55 °C as the annealing temperature. The PCR analysis was performed at Lifesequencing-ADM (Valencia, Spain).

2.2.2. Illumina Sequencing of 16S V3-V4 and 5.8S rDNA-LSU Amplicons

PCR amplification products of variable regions V3 –V4 in the 16S rRNA gene were obtained with fusion primers S-D-Bact-0341b-S-17 (Illumina adaptors + 5′ CCTAC-GGGNGG CWGCAG 3′) and S-D-Bact-0785-a-A-21 (Illumina adaptors + 5′ GAC-TACHVGGGTATCT AATCC 3′). PCR amplification products of the ITS3-ITS4 region were obtained with fusion primers ITS3F (Illumina adaptors + 5′ GCATCGATGAA-GAACGCAGC 3′) and ITS4R (Illumina adaptors + 5′ GCATATCAATAAGCGGAG-GA3′).

Amplicon multiplexing and sequencing was carried out using a dual indexing tag-tailed design with 8-nt indices, with the Nextera XT Index Kit v2. Paired-end sequencing

of 16S and ITS PCR amplicon libraries was performed with the MiSeq Reagent Kit v3, and 600 cycles were performed to produce 300 paired end sequences and the Illumina MiSeq. Library preparation and sequencing was performed at Lifesequencing-ADM (Valencia, Spain).

2.2.3. Bioinformatic Analysis

The 16S and ITS rRNA raw sequence data were first demultiplexed and then processed using PEAR V.0.9.1 (http://www.exelixis-lab.org/web/software/pear; Accessed: 6 October 2020) with default parameters, except for the overlap between the sequences of each end, which was fixed at 70 nts. This finally produced a single file with all overlapping sequences. Trimming steps were applied to remove adaptors and low-quality and short sequences. Primers for the target overlapping sequences were trimmed using CUTADAPT v.1.8.1 software. Sequences with quality values lower than Phred Q20 were excluded using BBMap version 3.38 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmap-guide/; Accessed: 6 October 2020). Finally, only sequences longer than 200 nts were retained, as shorter-length sequences are more likely to be incorrectly assigned to certain taxonomic groups.

For 16S rRNA only, chimeric reads were identified and excluded using Chimera UCHIME53 [43]. High-quality reads were taxonomically identified using operational taxonomic units (OTUs), and samples were assigned to the same group when reads showed 97% similarity (clustering at 97% homology was carried out using CD-HIT; Fu et al., 2012).

Based on the OTU approach, the alpha-diversity of the biofilm samples was estimated in terms of community richness (Chao-1) and diversity (Shannon). The longest read of each cluster was used as a reference for taxonomic classification, which was conducted using a local BLAST search with the blast-2.2.26+ algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Accessed: 6 October 2020), default settings and the NCBI database for 16s rRNA. In the final step, the taxonomic path of the reference sequence from each cluster was mapped to the additional reads within the corresponding cluster plus the corresponding replicates (as identified in the previous analysis step) to finally produce (semi-) quantitative information (number of individual reads representing a taxonomic path). Alpha and Beta diversity were measured using the VEGAN package in R. This analysis was performed by Lifesequencing-ADM.

The diversity of bacteria, fungi and Chlorophyta (green algae) on the N- and W-facing walls of the churches studied was evaluated using the Shannon index [44].

2.3. Statistical Analysis

Analysis of variance was applied using SPSS Statistics v19.0 (IBM) software to statistically determine any significant differences. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

The microbial diversity of granite cultural heritage buildings was analyzed in this work. Both traditional techniques and NGS techniques were used. Traditional culture-dependent techniques allowed the study of phototrophic organisms, such as algae and cyanobacteria, which are easily distinguished and examined by optical microscopy. Other abundant microorganisms in biofilms, such as bacteria and fungi were characterized by NGS techniques, which allow a complete study of those species that are impossible to culture in the laboratory. The combination of both methods made it possible to analyze organism diversity and to study the role of orientation and exposure to climatic factors in the microbiome composition.

3.1. Culture Dependent

Green algae and cyanobacteria were isolated from the three granite churches using a culture-dependent approach. The species/taxon compositions of the sampled biofilms are shown in Table 1 and the most representative species are shown in Figure 2. Four species

(*Trentepohlia* cf. *umbrina*, *Desmococcus olivaceus*, *Tychonema* cf. *bourrellyi*, and *Phormidium autumnale*) predominated in the churches, although differences in the abundance of the species were observed in the three churches and on the N- and W-facing walls of the same church. The N-facing walls of the SMV and BAR churches were dominated by *Trentepohlia* cf. *umbrina*, while the W-facing walls were predominated by *Desmococcus olivaceus* (Figure 3). In the ROC church, the cyanobacteria *Tychonema* cf. *bourrellyi* and *Phormidium autumnale* predominated on the W-facing wall, with a minor presence of *Desmococcus olivaceus*. Green algae (Chlorophyta) and cyanobacteria were similarly represented on the N-facing wall, with *Desmococcus olivaceus and Phormidium autumnale* predominating. Thus, the microbial communities on the SMV and BAR churches were similar to each other, but different from those on the ROC church (Figure 4). The biofilms on the first two churches were mainly composed of green algae, while biofilms on the ROC church contained a higher percentage of cyanobacteria, especially on the W-facing wall.

Table 1. Species composition of the phototrophic communities on the SMV, BAR, and ROC churches, expressed as relative frequencies (%) of the total number of cells observed.

Species		NORTH			WEST		
		SMV	BAR	ROC	SMV	BAR	ROC
Trentepohlia cf. umbrina	Chl	80	75		10	23	
Desmococcus olivaceus	Chl	9	10	53	81	71	15
Phormidium autumnale	Cy		1	40			30
Tychonema cf. bourrellyi	Cy	1					50
Bracteacoccus minor	Chl	1			1		
Chlamydomonas sp. sensulato	Chl		1				
Coccomyxa confluens	Chl	1		1	1	1	
Coenochloris signiensis	Chl	1	1				
Dictyosphaerium chlorelloides	Chl		1				
Diplosphaera chodatii	Chl	1	1	1	1	1	
Elakatothrix gelatinosa	Chl		1				
Cylindrocystis brebissoni	Chl		1				
Elliptochloris sp.	Chl	1	1	1	1	1	1
Gloeocystispoly dermatica	Chl	1	1				
Hantzschia amphyoxis	Ba		1				
Klebsormidium cf. flaccidum	Chl	1	1	1	1	1	1
Mesotaenium cf. caldariorum	Chl		1				
Pseudochlorella sp.	Chl	1	1		1	1	1
Stichococcus bacillaris	Chl	1		1			1
Stichococcus mirabilis	Chl	1	1		1		
Achnantidium cf. minutissimum	Ba		1				
Chlorella vulgaris	Chl				1		
Chroococcus turgidus	Cy				1		1
Nostoc punctiforme	Cy			1			
Oscillatoria tenuis	Cy					1	
Phormidium favosum	Cy		1				

Chl: Chlorophyta; Ba: Bacillariophyta; Cy: Cyanophyta.

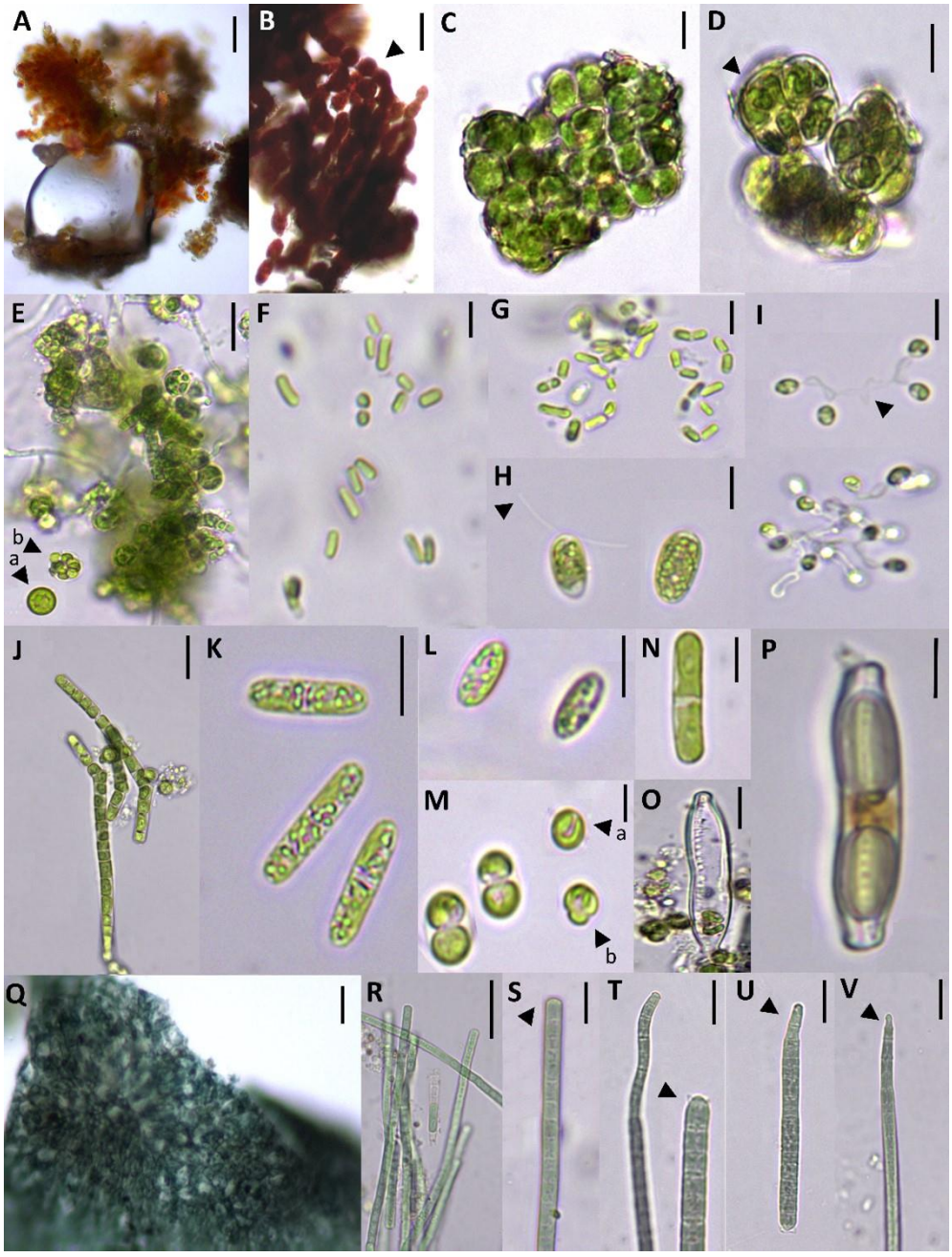

Figure 2. Species/taxa identified in the different samples observed on the surface of the north- and west-facing walls of the SMV, ROC and BAR churches. Chlorophyta or green algae (**A**–**N**): (**A**,**B**) *Trentepohlia* cf. *umbrina* (the arrow indicates the characteristic constriction of the filament cells); (**C**,**D**) *Desmococcus olivaceus*, genus appearance and cell details (arrow indicates central chloroplast pyrenoid); (**E**) *Bracteacoccus minor* (arrow indicates cell details and autospore); (**F**) *Stichococcus bacillaris*; (**G**) *Stichococcus mirabilis*; (**H**) *Chlamydomonas sp.* (arrow indicates one of the flagella); (**I**) *Dictyosphaerium chlorelloides* (Nauman) (arrow indicates mucilaginous tracts between cells); (**J**) *Klebsormidium* cf. *flaccidum* (Kützing); (**K**) *Mesotaenium* cf. *caldariorum*; (**L**) *Elakatothrix gelatinosa*; (**M**) *Chlorella vulgaris* (the arrows indicate in detail the morphology of the chloroplast (**A**) and the four-celled autospore (**B**)); (**N**) *Cylindrocystis brebissoni*; Bacillariophyta or diatoms: (**O**,**P**) *Hantzschia amphyoxis*, detail of siliceous frustule (**O**) and living cell (**P**); Cyanophyta (**Q**–**V**) (arrows indicate characteristic terminations of trichomes): (**Q**) Cyanobacterial film found on ROC church walls; (**R**,**S**) *Tychonema* cf. *bourrellyi*; (**T**) *Oscillatoria tenuis*; (**U**) *Phormidium autumnale*; (**V**) *Phormidium favosum* (Scales: **A**–**C**,**J**,**Q**,**R** = 20 µm; **D**–**I**,**K**–**N**,**S**–**V** = 10 µm; **O**–**P** = 5 µm).

Figure 3. Example of dominance of *Trentepohlia* cf. *umbrina* on the N-facing wall of the BAR church.

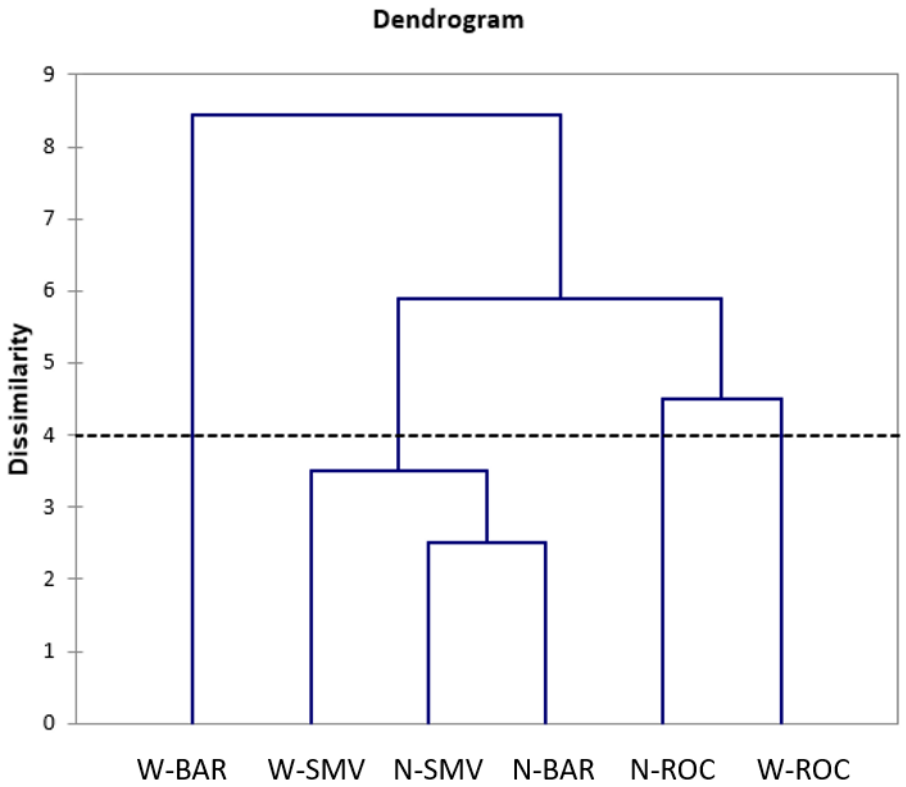

Figure 4. Agglomerative hierarchical analysis of the phototrophic component of the subaerial biofilm samples according to species composition. Dashed line indicates the cut-off point in the clustering of samples.

The species *Trentepohlia* cf. *umbrina*, *Desmococcus olivaceus*, *Stichococcus bacillaris*, and *Klebsormidium* cf. *flaccidum* were previously found to be the most representative dominant species on the exterior granite walls of the Galician Monumental Built Heritage, with *Trentepohlia* spp. characteristically found on N-facing walls [31,38], as in the SMV and BAR churches.

The dominant species were accompanied by others, mainly green algae (Chlorophyta), with a low representation and were identified in in vitro culture (Table 1). The higher species diversity on the N-facing wall of the BAR church was noteworthy. In addition, the presence on that wall of diatom species (*Bacillriophyta*), such as *Hantzschia amphyoxis* and *Achnantidium* cf. *minutissimum*, which lack desiccation resistance mechanisms, and liquid water-loving flagellate motile species, such as *Chlamydomonas sp. sensu lato*, clearly indicates increased water availability at the stone surface.

3.2. Next Generation Sequencing

Microbial community composition (of both bacteria and fungi) of the samples collected from both N- and W-facing walls of the SMV, BAR and ROC churches is represented in Figure 5A (at the phylum level) and Figure 5B (at the class level). The biofilms sampled include a large diversity of species, particularly in the bacterial domain, but also with a strong presence of lichenized fungi. The main species present are shown in Table 2. The value of sampling rural churches to analyze the biodiversity of granite colonizing organisms was previously reported [31,45] and the substrate aspect, inclination, wetness, exposure to rain, degree of disaggregation of the rock, and proximity to mortar were indicated as important factors in determining the composition of the flora.

Bacteria, which are known to discolor, disrupt, weaken and dissolve a wide variety of materials [46], proved the most diverse domain. The main phyla of bacteria that were found, which in several of the churches accounted for up to 50% of the colonization, were Proteobacteria and Bacteroidetes, followed by Acidobacteria, Plantomycetes, and Actinobacteria. The phyla Deinococcus-Thermus, Verrucomicrobia, Armatimonadetes and Cyanobacteria were also present, but were less abundant.

Within the phylum Proteobacteria, the most abundant class was Alphaproteobacteria, with Sphingomonadales and Rhodospirillales being the most abundant orders on most of the walls and with fewer representatives of Caulobacterales, Rhizobiales and Rhodobacteralesorders. Sphingomonads have been reported in a wide diversity of rocky habitats, ranging from a preserved sandstone slab at an exposed site in Oxford, UK [47], to the stone surfaces of monuments on the UNESCO World Heritage List of the Hangzhou West Lake Cultural Landscape in China [48]. The most abundant genus of the order Sphingomonadales found on the church walls was *Sphingomonas* (including the species *Sphingomonas prati, Sphingomonas hengshuiensis* and *Sphingomonas echinoides*). It was identified from biofilms from the N-facing walls of the SMV and BAR churches (31.0 and 15%), as well as from the W-facing walls of SMV, BAR and ROC (4.9, 20.1 and 10.25%, respectively). Species of the genus *Sphingobium* were also present on the W-facing wall of the BAR church (8.3%), and species of the genus *Sphingoaurantiacus*, specifically *Sphingoaurantiacus polygranulatus* was also found in the N- and W-facing wall of BAR (1.3 and 11.3%) and in the N-facing wall of SMV (8.4%). These genera are often regular inhabitants of heritage walls, due to their ability to live in nutrient-poor environments. Their presence causes a visual deterioration of the heritage due to the change of color that their presence produces in the stone, as well as the production of sphingans, a rather viscous exopolysaccharide [49–52].

Figure 5. Relative abundances (%) of bacterial and fungi group for each phylum (**A**) and class (**B**). Only species with a frequency greater than 0.001% were considered in the analysis.

All genera of the second most abundant order in the studied biofilms, Rhodospirillales, are part of the so-called acetic acid bacteria. This order was particularly abundant on the N-facing wall of the ROC church, where the genera *Gluconacetobacter* and *Granulibacter* occur (5.7 and 12.4%). The species *Roseomonas arcticisoli* (5.7%) and *Dankookia rubra* (1%) were identified on the N-facing wall of the SMV church.

Other less abundant species of this order, such as *Acidicaldus organivorans*, occurred on the N- and W-facing walls of the BAR church (2.3 and 6.7%), as well as on the N-facing wall of the SMV church (5.2%) and on W-facing wall of the ROC church (1%). Moreover, *Roseomonas riguiloci*, *Acidisphaera rubrifaciens* and *Acidisoma sibiricum* also colonized the N-facing wall of the ROC church (4.1, 1.5 and 3%), but also the W-facing wall of this church (5%), the W-facing wall of the BAR church (1.2%) and the N-facing wall of the SMV church (1%). These species are highly tolerant of low pH conditions, which is advantageous to growth on granitic substrate [53,54] because the substrate acidity is a key factor determining which organisms will develop most successfully. In terms of biodeterioration, some consideration should be given to *Acidicaldus organivorans* as its sulfide-reducing nature contributes to mineral dissolution [55].

Table 2. Species composition and taxonomic classification of the main organism conforming the cultural heritage microbiome identified by NGS. Pink background corresponds to Bacteria phylum, while the brown background corresponds to Fungi kingdom.

DOMAIN	PHYLUM	CLASS	ORDER	NORTH (%)			WEST (%)			GENUS	SPECIES
				SMV	BAR	ROC	SMV	BAR	ROC		
BACTERIA	Proteobacteria	Alphaproteobacteria	Caulobacterales	1.2	2.4	7.9	11.0	1.3	1.9	*Brevundimonas*	*Brevundimonas albigilva*
											Brevundimonas variabilis
										Phenylobacterium	*Phenylobacterium aquaticum*
			Rhizobiales	1.4	6.0	10.3	1.6	2.4	8.7	*Salinarimonas*	*Salinarimonas rosea*
										Bauldia	*Bauldia litoralis*
										Roseiarcus	*Roseiarcus fermentans*
										Aureimonas	*Aureimonas sp.*
			Rhodospirillales	14.4	6.3	29.4	1.0	10.5	10.6	*Acidicaldus*	*Acidicaldus organivorans*
										Gluconacetobacter	*Gluconacetobacter sp.*
										Granulibacter	*Granulibacter sp.*
										Roseomonas	*Roseomonas arcticisoli*
											Roseomonas riguiloci.
										Acidisphaera	*Acidisphaera rubrifaciens*
										Acidisoma	*Acidisoma sibiricum*
										Dankookia	*Dankookia rubra*
			Sphingomonadales	41.9	17.4	3.3	16.5	40.8	12.0	*Sphingobium*	*Sphingobium sp.*
										Sphingomonas	*Sphingomonas prati.*
											Sphingomonas hengshuiensis
											Sphingomonas echinoides
										Sphingoaurantiacus	*Sphingoaurantiacus polygranulatus*
		Betaproteobacteria	Burkholderiales	/	1.8	0.8	0.1	/	2.3	*Massilia*	*Massilia sp.*
		Deltaproteobacteria	Myxococcales	0.3	1.5	0.3	/	/	0.2	*Labilithrix*	*Labilithrix luteola*

Table 2. *Cont.*

DOMAIN	PHYLUM	CLASS	ORDER	NORTH (%)			WEST (%)			GENUS	SPECIES
				SMV	BAR	ROC	SMV	BAR	ROC		
BACTERIA	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	/	11.0	2.6	5.4	1.5	22.5	*Arcticibacter*	*Arcticibacter svalbardensis*
										Pedobacter	*Pedobacter tournemirensis*
		Cytophagia	Cytophagales	/	17.5	/	5.0	9.0	6.3	*Spirosoma*	*Spirosoma rigui*
										Siccationidurans	*Siccationidurans ginsengisoli*
		Flavobacteriia	Flavobacteriales	/	0.1	/	16.6	/	/	*Pricia*	*Pricia antarctica*
										Flavimarina	*Flavimarina pacifica*
	Acidobacteria	Acidobacteriia	Bryobacteraceae	0.8	2.5	3.1	0.4	0.7	0.8	*Paludibaculum*	*Paludibaculum fermentans*
			Acidobacteriales	14.9	6.2	23.5	1.0	1.2	21.3	*Granulicella*	*Granulicella acidiphila*
											Granulicella mallensis
										Silvibacterium	*Silvibacterium bohemicum*
										Edaphobacter	*Edaphobacter modestus*
										Bryocella	*Bryocella elongata*
										Terriglobus	*Terriglobus aquaticus*
											Terriglobus aquaticus
											Terriglobus roseus
	Planctomycetes	Planctomycetia	Planctomycetales	12.7	4.0	4.2	/	2.7	3.2	*Aquisphaera*	*Aquisphaera giovannonii*
										Paludisphaera	*Paludisphaera borealis*
										Singulisphaera	*Singulisphaera rosea*
		Phycisphaerae	Tepidisphaerales	/	/	/	/	2.7	/	*Tepidisphaera*	*Tepidisphaera mucosa*
	Actinobacteria	Actinobacteria	Rubrobacterales	0.1	/	/	14.5	5.8	/	*Rubrobacter*	*Rubrobacter radiotolerans*
			Kineosporiales	/	/	/	2.7	0.1	/	*Pseudokineococcus*	*Pseudokineococcus lusitanus*
			Actinomycetales	0.3	0.3	/	5.6	0.5	/	*Janibacter*	*Janibacter sp.*
										Serinicoccus	*Serinicoccus sp.*

Table 2. *Cont.*

DOMAIN	PHYLUM	CLASS	ORDER	NORTH (%)			WEST (%)			GENUS	SPECIES
				SMV	BAR	ROC	SMV	BAR	ROC		
BACTERIA	Deinococcus-Thermus	Deinococci	Trueperales	0.2	0.2	/	2.4	2.9	/	*Truepera*	*Truepera radiovictrix*
	Verrucomicrobia	Spartobacteria	Cthnoniobacterales	/	1.5	0.2	/	2.3	1.0	*Chthoniobacter*	*Chthoniobacter flavus*
	Cyanobacteria	Cyanophyceae	Nostocales	/	/	/	/	/	0.2	*Loriellopsis*	*Loriellopsis cavernicolae*
			Synechococcales	/	/	/	/	/	0.4	*Trichocoleus*	*Trichocoleus desertorum*
FUNGI (EUKARYA)	Ascomycota	Lecanoromycetes	Lecanorales	6.7	23.0	2.1	52.9	26.1	42.2	*Hypogymnia*	*Hypogymnia metaphysodes*
										Scoliciosporum	*Scoliciosporum umbrinum*
										Lecania	*Lecania cyrtella*
											Lecania erysibe
										Lecanora	*Lecanora dispersa*
											Lecanora horiza
			Pertusariales	0.9	6.6	0.2	/	0.5	/	*Lepra*	*Lepra amara*
			Teloschistales	/	1.0	/	0.2	14.7	/	*Caloplaca*	*Caloplaca maritima*
		Dothideomycetes	Capnodiales	22.3	10.0	24.5	13.4	4.1	10.6	*Catenulostroma*	*Catenulostroma protearum*
										Neodevriesia	*Neodevriesia lagerstroemiae*
										Devriesia	*Devriesia strelitziicola*
		Eurotiomycetes	Chaetothyriales	27.5	17.4	0.8	14.5	16.4	16.7	*Unclas. Herpotrichiellaceae*	*Herpotrichiellaceae sp. MUT 5408*
										Rhinocladiella	*Rhinocladiella sp.*
										Cladophialophora	*Cladophialophora sp.*
										Knufia	*Coniosporium sp. MA 4597*
	Basidiomycota	Agaricomycetes	Cantharellales	/	7.8	/	4.4	1.7	/	*Rhizoctonia*	*Rhizoctonia sp.*
		Tremellomycetes	Tremellales	3.4	0.4	1.1	3.5	/	1.0	*Fellomyces*	*Fellomyces sp.*

Within the order Caulobacterales, the species *Brevundimonas albigilva* was found to colonize the N-facing walls of the BAR and ROC churches (1.4 and 2.8) while *Brevundimonas variabilis* only appeared on the N-facing wall of the ROC church (11%). *Phenylobacterium aquaticum* was found on the N-facing walls of the SMV and ROC churches (1 and 4.6%), as well as on the W-facing wall of the ROC church (1.2). Both genera have previously been found colonizing other monuments, especially on sandstone in polluted areas, as they can survive in such environments due to their capacity to degrade hydrocarbons and use them as a carbon source [56–58].

Members of the order Rhizobiales mainly appeared on the ROC church. More specifically, *Salinarimonas rosea* was found on both the N- and W-facing walls of this church (3.2 and 1%), as well as on the N-facing wall of the BAR church (5.8%). In addition, *Bauldia litoralis* and *Roseiarcus fermentans* were also found on the ROC church, the former on both N- and W-facing walls (4 and 1.5%) and the latter on the N-facing wall (4.1%). This species produces bacteriochlorophyll *a,* and carotenoids, which have been reported to cause an aesthetic impact on the stone where the species occurs [59]. Examples of the genus *Aureimonas* were only found on W-facing walls, in all three churches (approx. 2%). This order is commonly associated with the roots of vascular plants and its occurrence may indicate an advanced level of colonization on the façades (plants rooted in the rock) but may also be due to the influence of endophytes from neighboring forest areas deposited on walls by winds. It has been found to colonize a red sandstone monolithic statue of Buddha [53] in a region of China with a subtropical monsoon climate and a large presence of higher plants.

The other classes of Proteobacteria (Beta and Deltaproteobacteria) were present in much smaller proportions. Species of the order Burkholderiales were found on the N-facing wall of the BAR church and on the W-facing wall of the ROC church, where the genus *Massilia* appeared; members of the order Myxococcales were mainly distributed on the N-facing walls of the churches, along with members of the genus *Labilithrix*.

The most abundant classes within the phylum Bacteroidetes were Sphingobacteriia, Cytophagia and Flavobacteriia. Some widely distributed species, such as *Arcticibacter svalbardensis*, which belongs to the order Sphingobacteriales, were present on the N-facing walls of all the churches (SMV, BAR and ROC: 5, 5.6, 2.4% respectively) as well as on the west-facing wall of the ROC church (9%). Other species within the same order, e.g., *Pedobacter tournemirensis*, were very abundant, but only on the W-facing wall of the ROC church (11%). Within the order Cytophagales, different species of the genus *Spirosoma* were found on both the N-and W-facing walls of the BAR church, specifically *Spirosoma rigui* (3.5%), and *Siccationidurans ginsengisoli* was only found on the W-facing wall of the ROC church (5.6%). Finally, within the order Flavobacteriales, the species *Pricia antarctica* and *Flavimarina pacifica* (6.5 and 8.8%) occurred on the N-facing wall of the SMV church.

The most common orders in the phylum Acidobacteria were Solibacterales and Acidobacteriales. The former is mainly represented by one species, *Paludibaculum fermentans*, which was only found on the N-facing walls of BAR and ROC churches (1.3 and 3%). In the case of the order Acidobacteriales, the species *Granulicella acidiphila* was found on the N- and W-facing walls of the ROC church (11.7 and 11.6%), as well as on the N-facing wall of SMV (14%). This species is clearly acidophilic and is known to produce large amounts of EPS and carotenoids [60] which can be important in terms of biodeterioration. *Silvibacterium bohemicum* was found on both the N- and W-facing walls (8.3 and 1.5%) of the ROC church. Other species of the order, such as *Edaphobacter modestus, Granulicella mallensis* and *Terriglobus aquaticus*, were found in smaller percentages in the N-facing wall of the church of BAR, while *Bryocella elongata, Terriglobus aquaticus* and *Terriglobus roseus* were found on the W-facing wall of the ROC church.

Regarding the phylum Planctomycetes, only species belonging to two classes were found: Planctomycetia and Phycisphaerae. The first class has three different widely distributed species: *Aquisphaera giovannonii* was found at all sites at a percentage abundance of between 1 and 9%, except on the W-facing wall of the ROC church; *Paludisphaera borealis* was found on the N-facing walls of the SMV and ROC churches and on the W-facing wall

of the ROC church (between 1 and 3%); and *Singulisphaera rosea*, found on both the N- and W-facing walls of the BAR and ROC churches. The class Phycisphaerae was represented by a single species, *Tepidisphaera mucosa*, which occurred on the BAR church, with a percentage abundance of 2.5%, on both N- and W-facing walls.

The phylum Actinobacteria was mainly found on W-facing walls. The order Rubrobacterales was represented by species such as *Rubrobacter radiotolerans* on the W-facing wall of the SMV and BAR churches (5.7 and 2%). In addition, other species of the Rubrobacter genus were abundant on the SMV and ROC churches (between 1 and 9%). This genus has been widely linked to the biodeterioration of monuments and is known to contribute to the so-called rosy discoloration [61]. The pigment production is related to the high desiccation tolerance of this genus, which confers a selective advantage over other organisms [40]. Other orders such as Kineosporiales and Micrococcales were also abundant on the walls of the SMV church.

In relation to the less abundant phyla, Deinococcus-Thermus, which is associated with tolerance to high radiation and desiccation [50], was only represented by one species, *Truepera radiovictrix*, on the W-facing walls of the SMV and BAR churches (2.3 and 2.9%). The phylum Verrucomicrobia was represented by the species *Chthoniobacter flavus*, on both N- and W-facing walls of the BAR and ROC churches (around 1%). However, the phylum Cyanobacteria was very scarce, and the only species identified were *Loriellopsis cavernicolae* and *Trichocoleus desertorum*, both at abundances of less than 0.5%, on the W-facing wall of the ROC church. In the present study, Cyanobacteria seemed to be rather scarce, in contrast to the results of other studies investigating the diversity of microbial species found in cultural heritage formed by bricks, marbles [48], sandstones [62] and limestones [63], in which cyanobacteria constituted an important fraction of the community. Across Europe, much lower numbers of cyanobacteria are found growing on granite and sandstone than on other types of stone such as marble and limestone [12], which may indicate a preference of these species for alkaline substrates. In this study, the cyanobacteria most frequently detected by NGS was *Loriellopsis cavernicola*, which was previously found in caves in Mediterranean environments [64,65]. The ability of this species to mobilize calcium ions has been previously described [66] and may favor degradation of the substrate.

In relation to the Fungi kingdom (Eukaryota), the phyla most represented on the examined walls are the Ascomycota and Basidiomycota. Within the phylum Ascomycota, the most abundant classes were Lecanoromycetes, Dothideomycetes and Euromycetes, and the least abundant, Sordariomycetes. Lecanoromycetes is the main class conforming lichenized fungi. The most abundant species were *Lecania cyrtella*, which was only found on the W-facing walls of the SMV and BAR churches (51.5 and 19.6), *Scoliciosporum umbrinum*, which was found on the N-facing wall of the BAR church and the W-facing wall of the ROC church (16.5 and 45%), and *Hypogymnia metaphysodes*, which only colonized N-facing walls of the SMV and BAR churches (5.8 and 2.49). Other species of this phylum were also found on different walls of different churches, such as *Lecanora dispersa* (5%), *Lecania erysibe* (1%), on the W-facing wall of the BAR church, and *Lecanora horiza* (2.6%) and *Lepra amara* (5%), on the N-facing wall of the BAR church. Finally, we found a very high percentage of *Caloplaca maritima* (14.3%) on the W-facing wall of the BAR church. *Hypogymnia metaphysodes, Lecanora dipersa, Schasporom umbirum* and *Lepra amara* have previously been found on stone monuments [45,67,68]: *Lecanora dispersa* and *Lepra amara* are included among the main lichens known to colonize Galician granite [9,45] and have been associated with acidic substrates and cold and humid climates [69]. However, the usual habitat of *Lecania cyrtella*, which is commonly present on the walls of these churches, is wood, and proliferation of this species may be influenced by the presence of surrounding vegetation. Comparison of the effect of the orientation showed that Lecanoromycetes was less abundant on N-facing walls than on W-facing walls. This may be due to the fact that some lichenized fungi have a great capacity to withstand very exposed conditions [70], which may lead to successful colonization of walls that are particularly exposed to solar radiation, unlike the N-facing walls, which are always more humid and shaded. In fact, biofilms samples were taken

from N- and W- facing walls of these churches, because the south- and east- facing walls, which are more exposed to solar radiation, were completely covered by lichens.

The main classes of fungi conforming the 'black fungi' identified in this study were the Dothideomycetes and Eurotiomycetes, which is consistent with the findings of previous studies [71–73]. In the Dothideomycetes class, the order Capnoidales occurred on N- and W-facing walls of all churches. *Catenulostroma protearum* occurred on the N-facing walls of all three churches (19, 3, 24% in SMV, BAR and ROC respectively) and on the W-facing wall of the ROC church (7%). *Neodevriesia lagerstroemiae* colonized both N-and W-facing walls of the SMV church (1 and 10.5%) and *Devriesia strelitziicola* colonized the N-facing walls of the SMV and BAR churches (1.8 and 5.6%). Within the class Eurotiomycetes, the most widely represented order is Chaetothyriales. Within this order, species from the family *Herpotrichiellaceae* were found to colonize the N- (12.6%) and W- (1.2%) facing walls of the SMV church and the N-facing wall of the BAR church (11%); species of *Rhinocladiella* colonized the N-facing wall of the SMV church (14.6%) and the W-facing walls of all three churches (3, 15 and 1%); species of *Knufia* colonized the W-facing wall of the SMV (10%) and ROC (2%) churches and species of the genus Cladophialophora colonized the N-facing wall of the BAR church (5.6%). The presence of species of the genera *Catenulostroma, Rhinocladiella*, and *Knufia (Coniosporium sp. MA 4597)* have previously been found to be linked to biodeterioration processes on stone monument façades [74], and its EPS linked with process of corrosion [75]. In fact the vast majority of rock-inhabiting fungi have the ability to penetrate intercrystalline, producing small holes (biopitting) or taking advantage of previous fissures [76–78]. They are also considered to be one of the most stress-tolerant eukaryotes known, in part due to EPS production and their thick cell walls [79].

It is also important to note the absence of ubiquitous genera such as *Aspergillus*, *Cladosporium*, *Penicillium*, or *Alternaria*, all of them widely described as members of the microbiome of monuments [80,81]. However, in the case of *Alternaria*, it is a genus more closely linked to Mediterranean environments, and its low presence has previously been reported for the northwestern part of the Iberian Peninsula [82], where the studied churches are located. In addition, *Aspergillus* and *Penicillium* spores have been reported to be more frequent in urban than in rural areas [83], such as the one in the study.

In relation to the phylum Basidiomycota, a low presence was detected on the ROC church. However, species of the *Rhizoctonia* genus (Agaricomycetes) were identified on N-and W-facing walls of the BAR church (7.3 and 1.6%) and on the N-facing wall of the SMV church (4.2%), and species of *Fellomyces* (Tremellomycetes) were found on the N- and W-facing walls of the SMV church (2%).

The presence of lichenized fungi and non-lichenized fungi must be taken into account in evaluating biodeterioration as several lichenized and non-lichenized fungi secrete a variety of acidic primary and secondary metabolites with chelating functions, thus affecting the integrity of the stone [16]. Excreted acids react with different ions in the substrate, leading to biocorrosion and formation of secondary mycogenic minerals [84]. Lichens mainly cause physical weathering of granitic rocks, by inducing disaggregation of surface grains and engulfing them in their thalli [8,9]. The chemical action of the lichens on the granite causes dissolution of the minerals by production of organic acids as well as by an increase in the time of contact between the rock and the attacking solution [7]. On the other hand, black fungi are known to possess thick walls and produce significant amounts of EPS as a method to reduce environmental stress. EPS production allows these organisms to survive extreme conditions, such as temperature fluctuations, water stress, UV radiation, or nutrient deficiency [85]; however, it leads to the deterioration of stone [86].

Thus, taking into consideration the organisms found on the three churches, although the total microbial diversity did not depend on the different locations, it did depend on the orientation of the walls and was highest on the N-facing walls. The values of the Shannon index (Figure 6) indicated that diversity was generally highest among the bacteria, followed by the fungi and green algae. However, regarding Bacteria diversity, no significant differences were found between locations or orientations ($p > 0.05$). In the case of fungi

and green algae, a greater diversity of these organisms was found on the N-facing walls ($p < 0.05$). However, the low diversity observed in the green algae group may be due to the use of the culture-dependent technique, which generally reduces the variability detected [22]. In terms of abundance, the orders of bacteria found in highest proportion were the Sphingomonadales and the Acidobacteriales. Most of the fungi present in the microbial consortium are lichenized fungi belonging to the order Lecanorales, although non-lichenized fungi from Capnoidales and Chaetothyriales orders are also present in large proportion. Lichenized fungi were distributed differently in relation to orientation, and they were more abundant on the W-facing walls. In the case of green algae, one of the two most abundant species, *Trentepohlia* cf. *umbrina*, mainly occurred on N-facing walls, while the other, *Desmococcus olivaceus*, was found on W-facing walls. Moreover, diatoms were only present in the microbial consortium on the N-facing wall of the BAR church, which confirms the damper conditions on this wall.

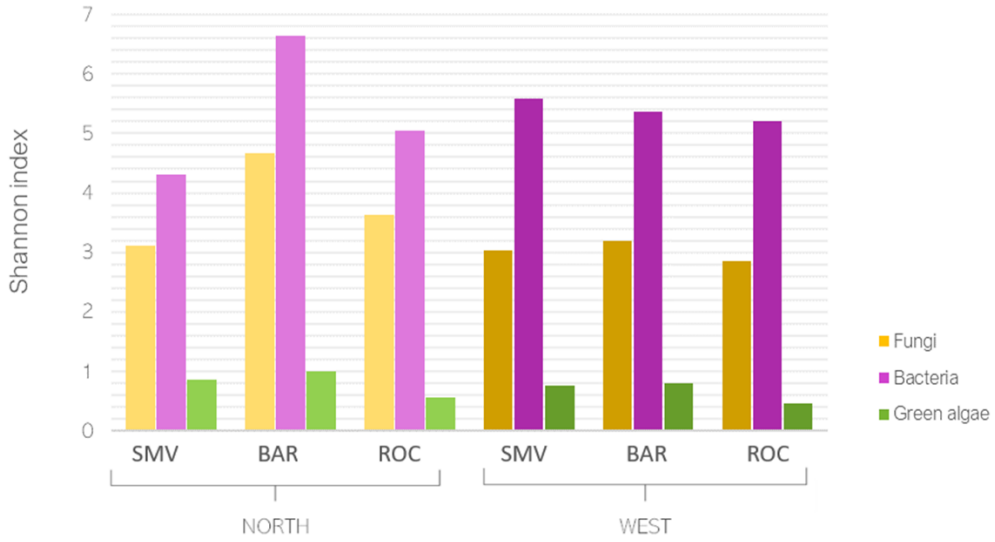

Figure 6. Shannon diversity index for green algae, fungi and bacteria colonizing the three churches under study (SMV, ROC, and BAR).

The low abundance of cyanobacteria on the church walls was noteworthy, especially relative to their presence in biofilms formed on other types of stone. Thus, for instance, cyanobacteria were the most abundant microorganisms in biofilms developed on limestone and marble [12,28]. However, the low abundance of cyanobacteria in the biofilms under study is consistent with the characteristics of biofilms on urban granite buildings in NW Spain [26]. In addition, they did not show any preferential distribution regarding orientation but did show a preferential distribution by location as they were only present on one church (ROC). The presence of cyanobacteria exclusively at ROC church may be due to the absence of close shade-producing structures, which are present in the case of the other churches. The absence of these structures results in a higher solar exposure of the west walls of ROC in relation to the west walls of SMV and BAR, favoring the development of cyanobacteria [87].

On comparing our results with those obtained by Vazquez-Nion et al. [26], who studied the microbial diversity in granitic urban heritage, we found that apart from bacteria (not considered in the aforementioned study), fungi and green algae were still the predominant groups in the biofilms. The groups most commonly present in the microbiota colonizing the churches were green algae (Chlorophyta) and Ascomycota (fungi), with cyanobacteria occurring in lower proportions. The diversity of fungi was very similar in both studies, with average Shannon index values of 3.42 (present study) and 3.43 (previous study).

Vazquez-Nion et al. [26] reported a higher diversity of algae, with Shannon index values between 1.75 and 1.54, in contrast to the values of less than 1 obtained in the present study.

4. Conclusions

The combination of culture-dependent techniques and next-generation sequencing enabled characterization, for the first time, of the microbial consortium present on historical granite buildings in rural areas. In the three buildings examined, the biofilms were mainly composed of bacteria, fungi (lichenized and non-lichenized), green algae and to a lesser extent cyanobacteria. Overall, this composition is very similar to that of biofilms on historical granite buildings in urban settings, especially in terms of fungal diversity. However, some differences were observed regarding the presence of algae, which were more abundant on urban buildings, and of lichenized fungi, which were more abundant in the rural environment.

Orientation also proved to be an important factor regarding both the diversity and abundance of microorganisms on the walls, with environmental factors associated with the north orientation favoring a higher diversity of fungi and green algae, and environmental factors associated with the west orientation determining the abundance of lichenized fungi. Among the two most abundant species of green algae, *Trentepohlia* cf. *umbrina*, mainly occurred on the N-facing wall, while *Desmococcus olivaceus* predominated on the W-facing wall.

These findings support the recommendation to characterize the microbial consortium forming the biofilms developed on the granite cultural heritage. The observed differences in both rural and urban environments, as well as regarding the orientation of different walls and the consequent exposure to different climatic factors, should be considered in selecting techniques to remove the colonizing microorganisms (such as the use of biocides), and when making any decisions related to the conservation of the buildings.

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