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Phototrophic Colonization in Dolomitic Limestone: Comparison between Single *vs* Artificial Multispecies

Fabiana Soares[a], João Trovão[a], Igor Tiago[a], Susana M. Cardoso[b], Francisco Gil[c,d], Lídia Catarino[e], and António Portugal[a,f]

[a]Department of Life Sciences, Centre for Functional Ecology, University of Coimbra, Coimbra, Portugal; [b]Department of Chemistry, LAQV-REQUIMTE, University of Aveiro, Aveiro, Portugal; [c]Department of Physics, Center for Physics, University of Coimbra, Coimbra, Portugal; [d]Department of Physics, Molecular Chemical-Physics Group, University of Coimbra, Coimbra, Portugal; [e]Department of Earth Sciences Geosciences Center, University of Coimbra, Coimbra, Portugal; [f]Fitolab-Laboratory for Phytopathology, Instituto Pedro Nunes, Coimbra, Portugal

ABSTRACT

Phototrophic organisms, such as microalgae and cyanobacteria, are known to be major contributors to stone decay. The purpose of this study was to assess the dolomitic limestone colonization by phototrophic organisms, using single *vs* artificial multispecies, under laboratory conditions. To achieve this aim, dolomitic limestone blocks were inoculated with single phototrophic species previously collected from the Old Cathedral of Coimbra, for a period of three months. In parallel, limestone blocks were also inoculated with a mixture of the same isolated single species, in order to compare the colonization capacities of both conditions. Results were evaluated based on visual inspection, surface covered area, colorimetric and SEM analyses. Results showed that the phototrophic organisms were able to colonize the dolomitic limestone blocks in both conditions (single *vs* artificial multispecies), but biofilm development was more enhanced when single species, rather than multispecies, were used. The obtained results also allowed to observe the capacity for endolithic colonization and the formation of small cavities by some species.

Introduction

Since the beginning of mankind, limestones, granites, and marbles have been extensively used for construction purposes, with preference being given to limestones, due to their ease to carve nature, pleasant appearance, and wide distribution across the globe (Dakal and Cameotra 2012; Miller et al. 2013). Nonetheless, as verified for other lithotypes, limestones can also be susceptible to deterioration caused by weather conditions, pollution, and colonizing organisms, such as fungi, bacteria, and/or phototrophic organisms (Scheerer et al. 2009). In fact, due to their photosynthetic nature, microalgae, and cyanobacteria can easily inhabit rock surfaces, either natural or handmade, being considered one of the major contributors to stone biodeterioration (Ascaso et al. 1998; Ascaso and Wierzchos 2002; Ortega-Morales 2006; Tomaselli et al. 2000). In this particular case, once successfully established on rock surfaces, phototrophic organisms can easily alter stone esthetics and physico-chemical properties (Gorbushina 2007). In fact, their simple occurrence on stone walls is considered a form of biodeterioration (Ortega-Calvo et al. 1995), as this term implies 'any undesirable change in the properties of a material caused by the vital activities of living organisms' (Hueck 1965). The adhesion and extent of colonization by phototrophic organisms is mediated by stone properties (color, roughness, porosity, mineral composition) and microclimatic conditions (availability of water, light, and organic matter) (Gaylarde 2020; Macedo et al. 2009; Sanmartín et al. 2020, 2021), and is, therefore, linked to stone bioreceptivity, which is define as 'the aptitude of a material to be colonized by one or several groups of living organisms without necessarily undergoing any biodeterioration' or as 'the totality of material properties that contribute to the establishment, anchorage and development of fauna and/or flora' (Guillitte 1995). According to Miller et al. (2012), all stone material is bioreceptive and, therefore, able to be colonized (at least to some extent).

The assessment of stone bioreceptivity and colonization can be inferred through laboratory-based experiments, by inoculating stone samples with colonizing microorganisms. Such studies may include one or more lithotypes inoculated with single or mixed species, incubated under optimal environmental conditions, to further quantify the resulting microbial biomass and its effect on the stone (Guillitte 1995; Miller et al. 2012). The most common organisms used in

this type of studies are phototrophic microorganisms (Miller et al. 2012) and the most commonly used methods to assess their biomass content are image analysis, chlorophyll *a* extraction, *in vivo* chlorophyll *a* fluorescence, and colorimetric analysis (De Muynck et al. 2009; Miller et al. 2010a, 2010b, 2012). As regards to the microorganisms used in such studies, some authors claim that the use of single species may not be as advantageous as the use of multispecies, since these latter can easily show the competition and/or synergy between colonizing organisms (Koestler et al. 1996). However, as far as it is known, there is still a lack of stone bioreceptivity and colonization studies comprising both single and artificial multispecies cultures, and a comparison between them. In this regard, the aim of this study was to assess, under laboratory conditions, the stone colonization to single phototrophic species and then compare it using the same single species as a mixture. For this purpose, dolomitic limestone blocks were artificially colonized with single phototrophic species previously collected from different biofilms in the Old Cathedral of Coimbra (UNESCO monument, Portugal) (see Soares et al. 2019a), and analyzed through stereomicroscopy, SEM and colorimetric measurements after three months of incubation. In parallel, additional blocks were also colonized using an artificial mixture of those single species, in order to compare both colonization processes using the same methods described above. To the best of our knowledge, apart from the green microalga *Bracteacoccus* sp., the other strains of this current work have never been used in bioreceptivity and colonization studies. Moreover, as far as we know, this is the first study that uses both single and mixed artificial phototrophic isolates to access stone colonization.

Materials and methods

Cultivation of phototrophic microorganisms

The phototrophic microorganisms used in this study were previously collected and isolated from different biofilms from the limestone walls of the Old Cathedral of Coimbra, Portugal (see Soares et al. 2019a, 2019b, 2021). From the collected phototrophic organisms, nine species were selected to proceed with the colonization studies, namely, *Acutodesmus bajacalifornicus*, *Bracteacoccus* sp., *Heterochlamydomonas inaequalis*, *Jenufa aeroterrestrica*, *Myxacorys almedinensis*, *Parakomarekiella sesnandensis*, *Polulichloris henanensis*, *Pseudochloris wilhelmii*, and *Pseudostichococcus monallantoides*. The strains have been maintained in laboratory, incubated in liquid BG_{11} culture medium, at $20 \pm 1\,°C$, under a 16: 8 h (light: dark) photoperiod (30–40 µmol photons m^{-2} s^{-1} irradiance). For the colonization experiments, a fresh subculture of each individual species was maintained incubated in the conditions described above, prior to their inoculation on the stone blocks. An artificial multispecies culture was also prepared by incubating the nine mentioned strains together in the same liquid culture and in the same conditions described above, prior to their inoculation on the stone blocks.

Laboratory-based colonization experiment

For the study of colonization by phototrophic organisms, dolomitic limestone replicas with similar characteristics to the limestone walls of the Old Cathedral of Coimbra were obtained from the old quarry 'Banhos secos' located in Coimbra (Catarino et al. 2019) and were cut into small blocks of $4\,cm \times 4\,cm \times 3\,cm$ dimensions. No treatment or polish were applied to the surface of the stone blocks and these were characterized by having the following characteristics: chemical composition of $\sim 50\%$ $CaMg(CO_3)_2$; 23–31% CaO, 16–21% MgO, 0.8–4% Fe_2O_3 and residual quantities of other oxides; apparent porosity of $13.4 - 19.6\%$; density of 2.46–$2.30\,g/cm^3$; and uniaxial compressive strength of 67–34.6 MPa (Catarino et al. 2019; Faim 2014; Manupella et al. 1981; Quinta-Ferreira et al. 1992).

Blocks were washed with sterile water and autoclaved at $120\,°C$ and 1 atm for 20 min prior to their use. Afterwards, the upper surface of each stone block was inoculated in the center with $200\,µL$ of each individual phototrophic species, in exponential growth phase, using a sterile pipette. In order to further compare the stone colonization to single *vs* artificial multispecies, additional stone replicates were inoculated with a $200\,µL$ mixture of the single species, as mentioned above, also in exponential growth phase. Each stone block was inoculated separately and in triplicate. A total of 30 inoculated stone blocks were placed inside propylene boxes (each box containing 3 individual blocks inoculated with each individual species, plus one box containing 3 individual blocks inoculated with the multispecies culture), and incubated at $20 \pm 1\,°C$, under a 16: 8 h (light: dark) photoperiod (30–40 µmol photons m^{-2} s^{-1} irradiance), for three months. Sterile distilled water was added when needed to the bottom of the propylene boxes, to ensure that a continuously humid environment was maintained and a biofilm formation was promoted. All conditions were close monitored throughout the three months of incubation and three control blocks (blocks without inoculum) were also maintained.

Evaluation of the phototrophic limestone colonization

In order to assess the extent of phototrophic colonization, image analysis through stereo and light microscopy was conducted to quantify the covered surface area of each stone block. Photographic records were performed for all stone blocks on the inoculation day, and every 30 days until the end of the experiment. The obtained photographic data were then processed using ImageJ software in order to measure the occupied surface area (cm^2) of each species on the stone blocks. Data were achieved by measuring the occupied surface area of each individual stone replica after three months of incubation. The mean values ($n = 3$) and standard deviation for each individual species after three months of incubation were then used to obtain a graphical data of the surface covered area for each species.

Scanning electron microscopy (SEM) was also conducted in rock fragments collected from the control blocks, and where growth and biofilm were observed (in the inoculated blocks), in order to study phototrophic growth and possible

stone alterations. Analyses were conducted using a TESCAN Vega3 SBH (TESCAN, Brno, Czech Republic) after the application of a gold/palladium (Au/Pd) coating using an SC7620 Mini Sputter Coater/Glow Discharge System, following the manufacturer's protocol.

Colorimetric analyses were conducted with a portable spectrophotometer CM-700d (Konica Minolta, Japan) with a 3 mm measuring aperture. Readings were taken at different zones on the surface of each individual stone block, in a total of 12 readings per species (total of 120 readings for the whole bioreceptivity experiment). Color readings were conducted on the inoculation day and after three months of incubation. CIELAB color parameters was applied in order to quantify the color of the stone surface by three parameters: L^* (light/darkness), a^* (red-green), and b^* (yellow-blue). Total color variation (ΔE^*) was obtained by using the CIE formula: $\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]1/2$ (Huertas et al. 2006; Prieto et al. 2020). Color reflectance was also inferred by using the mean of the means obtained for each species, on the inoculation day and after three months of incubation. Data regarding these analyses were based on a comparison with the control blocks (blocks without colonization).

Statistical analysis

Statistical analysis was performed in order to evaluate biofilm color differences between the inoculation day and three months later, through a One-way ANOVA. In addition, another statistical analysis was performed in order to evaluate differences in the surface covered area by each species after three months of incubation. A One-way ANOVA was performed, followed by a multiple comparison Tukey test. All analyses were conducted in Past 4.03 software (Hammer et al. 2001).

Results

Dolomitic limestone colonization of single vs artificial phototrophic multispecies

Results showed that, after three months of incubation, the dolomitic limestone blocks were bioreceptive to all single species inoculated, as well as for the artificial multispecies culture. All single species, plus the multispecies culture, were able to successfully colonize the stone blocks and promoted biofilm formation (Figure 1). Visually, when it comes to biofilm development, and comparing both conditions, it can be observed that biofilm formation was more enhanced when single species were used, rather than when the stone blocks were inoculated with a multispecies culture (Figure 1).

In terms of surface covered area (cm^2), measured using ImageJ software, results showed that, after three months, *Pseudostichococcus monallantoides*, *Polulichloris henanensis*, and the multispecies culture, were able to colonize a more extensive area. On the other hand, the species that colonized a less extensive surface area were *Parakomarekiella sesnandensis*, *Myxacorys almedinensis*, and *Heterochlamydomonas*

inaequalis (Figure 2). Statistical analysis corroborated these results, as differences revealed to be significant between *P. monallantoides* vs *H. inaequalis*; *P. monallantoides* vs *Bracteacoccus* sp.; *P. monallantoides* vs *Jenufa aeroterrestrica*; *P. monallantoides* vs *M. almedinensis* and between *P. monallantoides* vs *P. sesnandensis* (see Supplemental Material Table S1 and Supplemental Material Table S2).

Assessment of phototrophic biofilm growth through colorimetry and SEM analyses

Colorimetric analysis was conducted based on a total of 12 readings per species (120 total readings for the 30 stone blocks). Results are presented based on the mean of the means of each species and by incubation time (ie incubation day and after three months of incubation).

ΔE^*ab values reflect the biofilm development, and results showed that the highest ΔE^*ab values were obtained for stone blocks inoculated with *Bracteacoccus* sp. (39.17 CIELAB units), *Acutodesmus bajacalifornicus* (34.55 CIELAB units), and *Jenufa aeroterrestrica* (33.66 CIELAB units), whereas the lowest values were obtained for stone blocks inoculated with the multispecies culture (20.95 CIELAB units), *Pseudostichococcus monallantoides* (24.00 CIELAB units), *Pseudochloris wilhelmii* (24.51 CIELAB units), and *Polulichloris henanensis* (25.13 CIELAB units) (Table 1). When it comes to color reflectance, the graphics presented in Figure 3 are in accordance with the ΔE^*ab values, where a major discrepancy in the reflectance can be observed for blocks inoculated with *Bracteacoccus* sp., *A. bajacalifornicus* and *J. aeroterrestrica*, and a lower discrepancy was observed for blocks inoculated with *P. monallantoides*, *P. wilhelmii*, *P. henanensis*, and the multispecies culture (Figure 3).

Results regarding the statistical analysis, showed that biofilm color differences were statistically significant for the L^* color parameter for all organisms used in the study (Table 2). When considering the a^* and b^* color parameters, it could be verified that statistical differences were only observed for *Bracteacoccus* sp. and *J. aeroterrestrica*, and for *H. inaequalis*, *P. monallantoides*, and *M. almedinensis*, respectively (Table 2).

In terms of biofilm color, the majority of the blocks exhibited a relatively homogeneous green color throughout the experiment, except blocks inoculated with *A. bajacalifornicus*, *P. wilhelmii*, and *Myxacorys almedinensis*, which presented some color variations, changing from green to yellowish (Figure 1). In addition, some white spots denoting fungal and bacterial contamination were also verified on stone blocks inoculated with *J. aeroterrestrica* and *P. sesnandensis* (Figure 1).

SEM analysis was conducted in order to understand the possible stone alterations induced by the colonization of the phototrophic organisms, after three months of incubation. Results showed that blocks inoculated with *Heterochlamydomonas inaequalis*, *P. wilhelmii*, and *M. almedinensis* presented a mucilaginous matrix covering the surface of the stone blocks, and that *P. monallantoides*, *Bracteacoccus* sp. and *J. aeroterrestrica* were able to grow
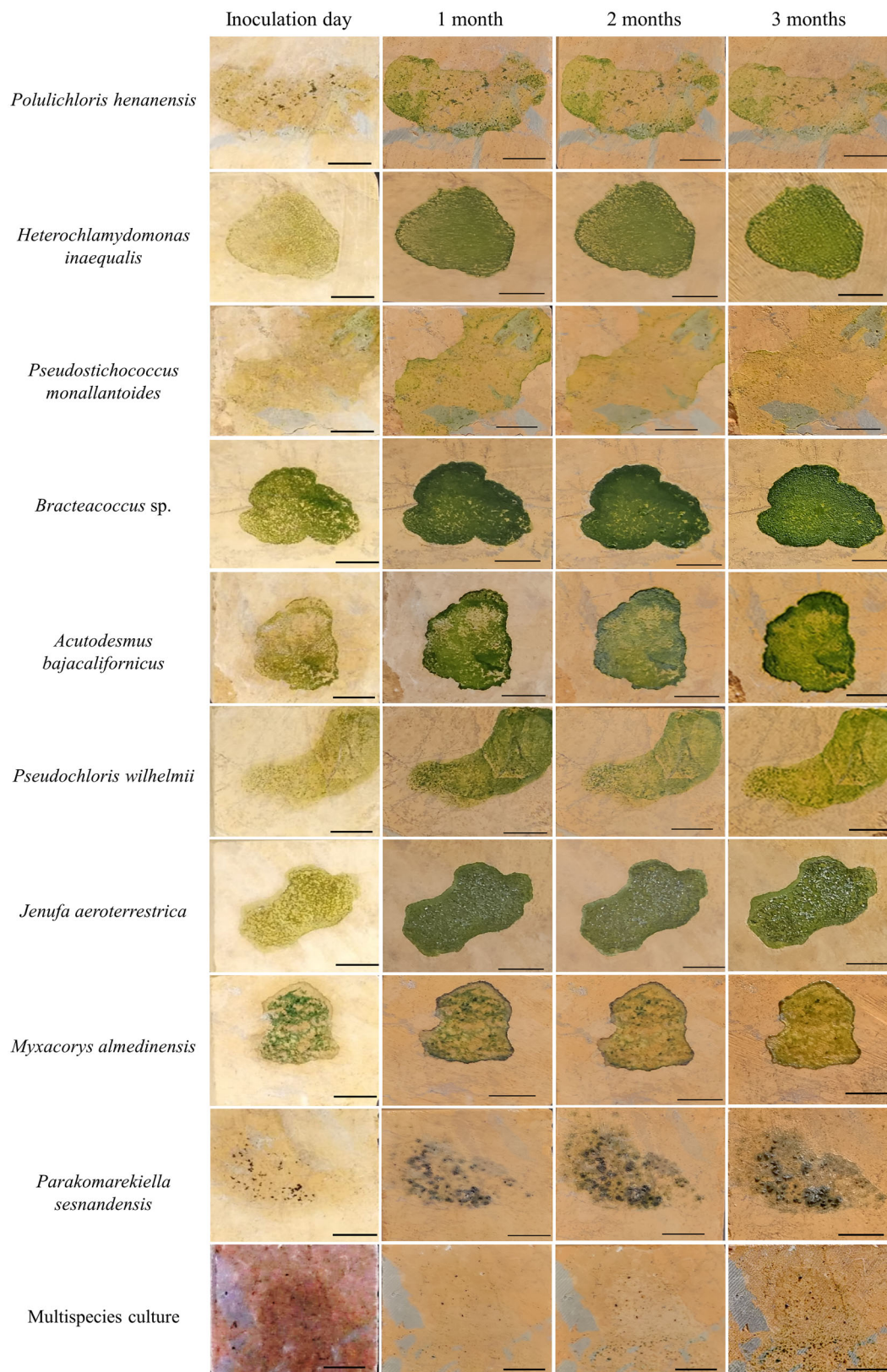
Figure 1. Phototrophic colonization on dolomitic limestone blocks during three months of incubation, under laboratory conditions. Reddish appearance (in the color version of this article) of the 'Multispecies culture' picture on the 'inoculation day' is due to the use of a different camera for that particular picture. Scale bars: 1 cm.

endolithically. This type of growth was also verified when stone blocks were inoculated with the multispecies culture. Interestingly, in stone blocks inoculated with *P. wilhelmii* and the multispecies culture, it was possible to verify small cavity formations, a phenomenon not observed in the other inoculated blocks. These small cavities were not observed in the uncolonized blocks (control blocks). Nevertheless, some of the organisms showed only an epilithical
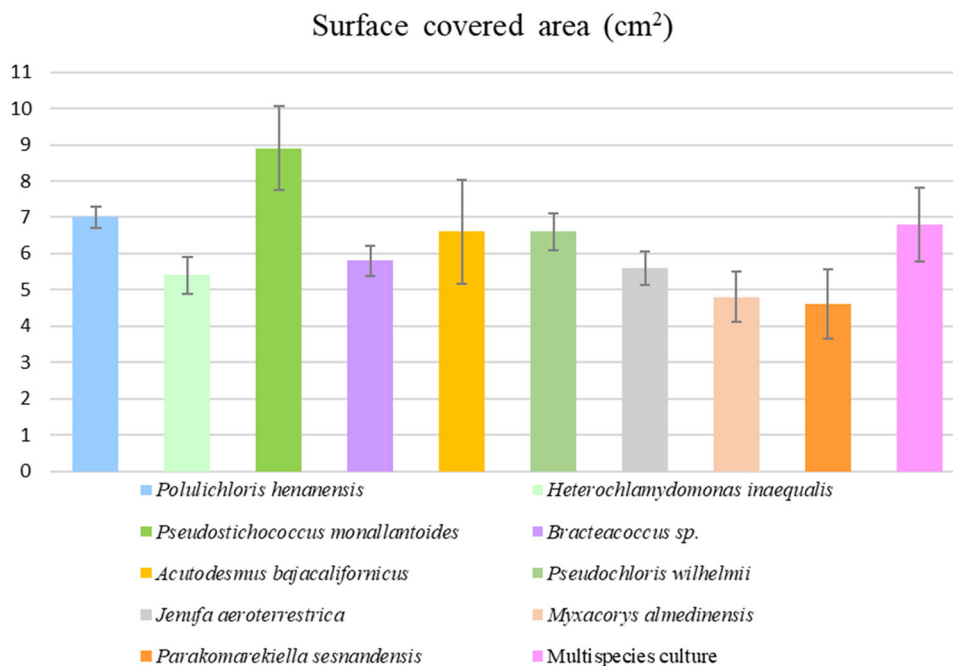
Surface covered area (cm^2)

Figure 2. Dolomitic limestone surface covered area (cm^2) by phototrophic organisms after three months incubation (n = 3; SD values).

colonization of the stone, such as *P. sesnandensis*. Some of these mentioned examples are presented in Figure 4.

Stereomicroscopy was used to visually inspect the stone blocks and the results are in accordance with what was observed in the SEM analysis. In stone blocks inoculated with *Bracteacoccus* sp., it can be observed that the phototrophic cells are intermixed with the stone matrix, denoting endolithic growth. On the stone blocks inoculated with *P. wilhelmii*, *J. aeroterrestrica*, *M. almedinensis,* and the multispecies culture, it can be observed an apparent mucilaginous matrix covering the surface of the stone blocks (Figure 5), corroborating the observations made by SEM, at least for some of the organisms.

Discussion

The aim of this study was to assess the capacity of the phototrophic organisms to colonize dolomitic limestone blocks, using single *vs* multispecies cultures, under laboratory conditions. Species used in this study were previously isolated from different biofilms found in the limestone walls of the Old Cathedral of Coimbra. This emblematic UNESCO monument was constructed using yellow dolomitic limestone, a sedimentary carbonate rock mainly composed by dolomite and calcite, with low magnesium and high iron contents (Carvalho et al. 2001; Manupella et al. 1981). It is known that some limestones, namely, Lioz, Dolomite, and Portunhos lithotypes, possess physical and chemical characteristics (ie resistance to compression and/or flexion and water absorption rate) that allow them to resist to the formation of cracks and fissures in harsh environments, where microorganisms usually find shelter (Carvalho et al. 2001; Pinheiro et al. 2019). Currently, it is widely accepted that the ability of a material to be colonized depends not only on its porosity, chemical composition, pH,

roughness, texture, and humidity (Guillitte 1995; Miller et al. 2012; Vázquez-Nion et al. 2018) but also on the color, architectural features, and surface temperature (Gambino et al. 2019; Gaylarde 2020; Sanmartín et al. 2020, 2021).

According to Miller et al. (2012) and Sanmartín et al. (2021), phototrophic organisms are the most commonly used microorganisms in laboratory-based bioreceptivity and colonization studies. To the best of our knowledge, this is the first study that uses both individual and multispecies phototrophic cultures to assess stone bioreceptivity and colonization, and to compare their colonization capacity when used alone or in a mixture. Although the use of single species may not be as advantageous as the use of multispecies, as these latter can more easily mimic the competition and/or synergy between colonizing organisms (Koestler et al. 1996), the use of single species is also of extremely importance, as it allows a more in-depth analysis on the microorganism-stone relationship and interaction at an individual level. In fact, it is considered that each individual species can play an important role in the first steps of biofilm formation, and that the same species can have the ability to cause different types of colonization (Marasco et al. 2016; Urzì et al. 2010). According to some authors, cyanobacteria are considered the primary inhabitants when considering outdoor exposed monuments, thanks to their photosynthetic nature and ability to fix nitrogen. In addition, the production of organic matter by these organisms can then contribute to future colonization by heterotrophic microorganisms (Albertano 2012; Crispim and Gaylarde 2005; Ortega-Calvo et al. 1991; Tiano et al. 1995). However, according to Mulec et al. (2008), although cyanobacteria are the most adaptable to harsh environments, areas with more favorable conditions can lead to an increase growth of microalgae. On the other hand, Gaylarde and Gaylarde (2000), stated that eukaryotic algae are the usual first colonizers of stone walls, with

Table 1. Quantification of phototrophic biofilm development by colorimetric analysis.

Organism	Inoculation day			Three months later			% variation			ΔE*
	L* (D65)	a* (D65)	b* (D65)	L* (D65)	a* (D65)	b* (D65)	L* (D65)	a* (D65)	b* (D65)	
Polulichloris henanensis	71.31 (±0.64)	5.4 (±2.14)	18.17 (±3.95)	47.41 (±2.34)	4.46 (±3.94)	25.88 (±2.51)	33.53 (±2.82)	40.57 (±73.78)	−46.44 (±19.02)	25.13
Heterochlamydomonas inaequalis	71.26 (±1.06)	5.95 (±1.16)	20.72 (±1.99)	43.93 (±5.81)	5.28 (±3.85)	30.93 (±3.27)	39.35 (±8.07)	18.40 (±54.92)	−51.52 (±27.76)	29.84
Pseudostichococcus monallantoides	71.51 (±1.03)	4.75 (±0.62)	17.36 (±0.42)	50.17 (±0.50)	7.53 (±3.06)	27.99 (±5.26)	29.83 (±0.89)	−53.26 (±51.49)	−60.69 (±26.97)	24.00
Bracteacoccus sp.	71.42 (±0.77)	5.43 (±0.51)	20.95 (±2.99)	33.10 (±2.43)	−2.62 (±1.28)	19.99 (±1.67)	53.64 (±3.55)	147.70 (±20.89)	2.71 (±14.76)	39.17
Acutodesmus bajacalifornicus	71.48 (±0.09)	6.85 (±0.51)	22.87 (±0.83)	37.94 (±5.61)	−0.93 (±3.83)	25.74 (±2.19)	46.91 (±7.90)	116.68 (±58.68)	−12.81 (±11.50)	34.55
Pseudochloris wilhelmii	69.85 (±0.92)	5.36 (±0.64)	20.15 (±2.50)	46.04 (±2.17)	4.78 (±1.99)	25.97 (±4.20)	34.09 (±2.82)	6.30 (±47.50)	−28.21 (±5.47)	24.51
Jenufa aeroterrestrica	71.77 (±0.81)	5.66 (±0.92)	19.82 (±1.36)	38.68 (±1.85)	−0.51 (±1.01)	20.24 (±2.39)	46.13 (±2.05)	111.73 (±19.14)	−2.75 (±14.51)	33.66
Myxacorys almedinensis	70.55 (±1.11)	6.26 (±0.61)	21.81 (±2.33)	44.06 (±0.83)	7.30 (±0.65)	28.74 (±0.40)	37.56 (±0.53)	−18.51 (±21.55)	−33.19 (±13.35)	27.41
Parakomarekiella sesnandensis	70.47 (±1.01)	6.26 (±0.65)	21.49 (±0.89)	42.20 (±5.40)	6.24 (±0.82)	18.70 (±1.67)	40.04 (±8.21)	0.54 (±3.36)	13.06 (±5.34)	28.41
Multispecies culture	71.69 (±0.90)	6.49 (±1.51)	22.66 (±3.18)	51.18 (±0.75)	8.27 (±2.52)	26.53 (±2.43)	28.58 (±1.95)	−28.37 (±24.26)	−18.55 (±12.52)	20.95

Notes: A total of 12 readings per species were measured (120 total readings for a total of 30 stone blocks). Color measurements were conducted on inoculation day and after three months.

cyanobacteria becoming predominant at later stages. Although some controversy regarding this topic seems to persist, it is known that nitrogen-fixing cyanobacteria are relevant for the establishment and development of heterotrophic organisms, such as bacteria and fungi (Crispim and Gaylarde 2005; Grant 1982). At later stages, mosses and other plants can also colonize these sites, forming complex communities with relevant trophic interactions (Jurado et al. 2020). In this current work, both cyanobacteria and microalgae were able to successfully colonize the dolomitic limestone blocks and to cause important biodeterioration phenomena in both experimental conditions (when colonizing individually or in a mixture). Nonetheless, in light of the results gathered during the course of this work, we were still unable to accurately state which one of these microorganisms (microalgae or cyanobacteria) are in fact the primary colonizers. More studies in this regard are need, specially with other heterotrophic bacteria and fungi as well, in order to better understand ecological theories regarding community succession and complexity.

In the overall, and as stated before, this study revealed that the dolomitic limestone blocks were bioreceptive to all individual phototrophic isolates, as well as to the multispecies culture, and that the phototrophic organisms were able to colonize the stone blocks when used alone or in a mixture. This could have been related to the fact that the experiment was conducted under laboratory conditions, where optimal conditions of light, temperature, and moisture were present. The creation of such perfect conditions can contribute to the colonization process, as well as to the formation and establishment of a biofilm. In fact, it is known that the presence of water is an important factor for microbial colonization, with microalgae growing best at 100% relative air humidity (Del Mondo et al. 2021; Häubner et al. 2006). In this sense, it is important to have in mind that bioreceptivity and colonization experiments under controlled conditions can be different than those performed in the field (in-situ), as the physico-chemical properties of a given substrate may change over time due to constant exposure to different abiotic conditions (eg, weather alterations, pollution, etc.) (Sanmartín et al. 2021).

In terms of development, results showed that biofilms were much more enhanced when single species, rather than multispecies, were used. This is reflected on the ΔE*ab values, as the lowest value was obtained for the stones inoculated with the multispecies culture. On the other hand, biofilms were more developed when stone blocks were inoculated with the green microalgae isolates of *Bracteacoccus* sp., *Acutodesmus bajacalifornicus,* and *Jenufa aeroterrestrica.* The lowest ΔE*ab value achieved for the multispecies culture could be due to the competition between all species and/or due to the detriment of growth of some species in regards to others, which is sometimes observed when enrichment cultures are established. In terms of comparison of ΔE*ab values, *Polulichloris henanensis, Pseudostichococcus monallantoides,* and *Pseudochloris wilhelmii* were the ones, along with the multispecies culture, that showed the lowest ΔE*ab values, which is in accordance with what can be
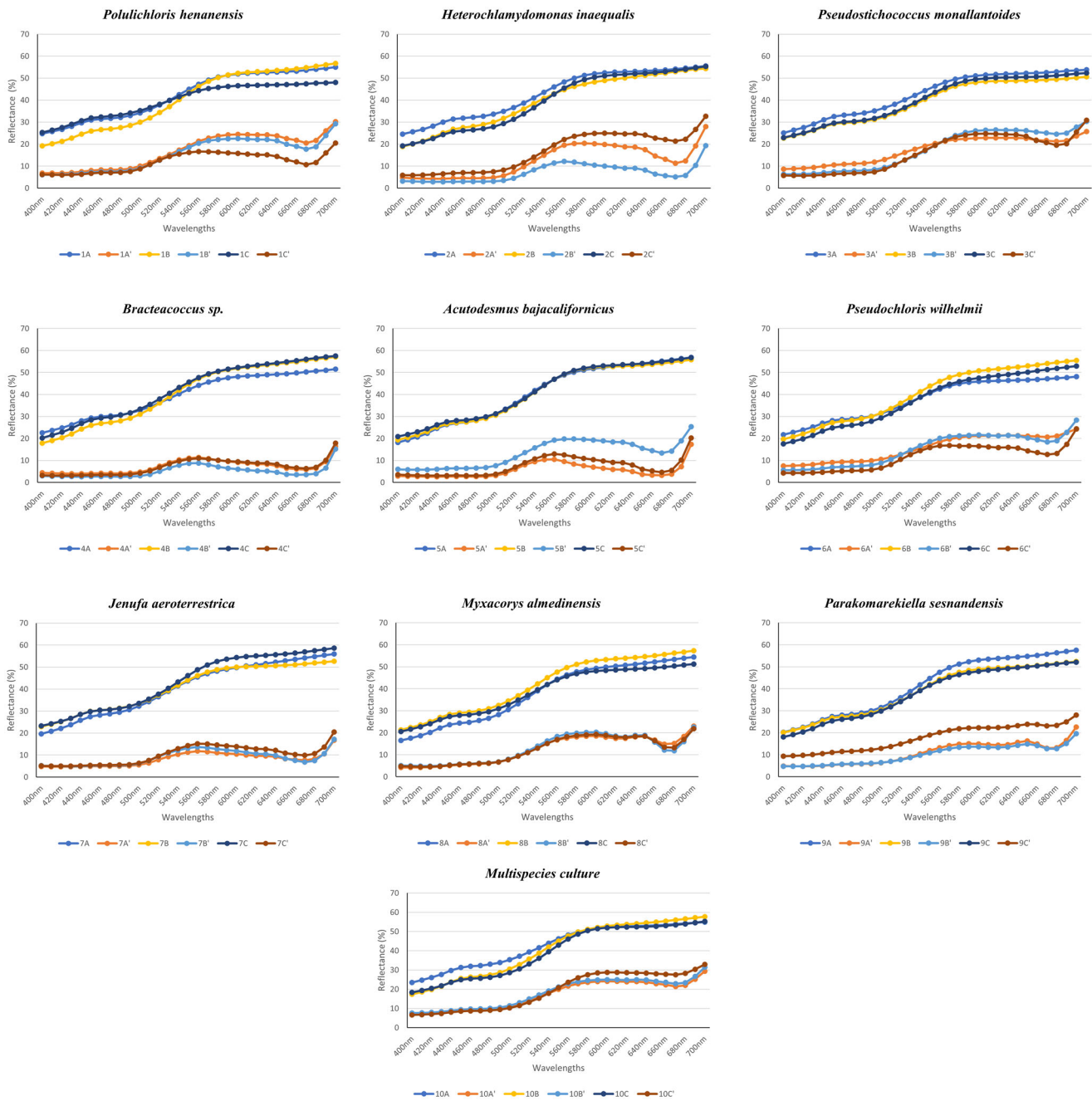
Figure 3. Color reflectance regarding phototrophic biofilm development on dolomitic limestone blocks. Letters A, B and C correspond to color reflectance at the inoculation day, whereas A', B' and C' correspond to color reflectance after three months. Results are presented based on the mean values of each replicate on the incubation day and after three months of incubation.

Table 2. Statistical differences regarding the CIELAB color parameters for each phototrophic organism used in the bioreceptivity study.

Organism	L* (D65)	a* (D65)	b*(D65)
Polulichloris henanensis	0.0001553	0.7794	0.08013
Heterochlamydomonas inaequalis	0.00256	0.827	0.01962
Pseudostichococcus monallantoides	0.00001238	0.2763	0.04627
Bracteacoccus sp.	0.00002863	0.00117	0.7117
Acutodesmus bajacalifornicus	0.001071	0.4632	0.158
Pseudochloris wilhelmii	0.0001385	0.7122	0.1674
Jenufa aeroterrestrica	0.00002074	0.003088	0.8396
Myxacorys almedinensis	0.0000113	0.1772	0.01436
Parakomarekiella sesnandensis	0.001893	0.9797	0.1052
Multispecies culture	0.00001609	0.439	0.2429

Note: Significant values ($p < 0.05$) are highlighted in grey.

verified visually. In addition, when particularly analyzing the three CIELAB color parameters (L*, a*, and b*), it was possible to verify that the L* parameter (which is related to lightness or luminosity of color), decrease for all blocks after three months of incubation. This clearly confirmed that the biofilms that grew on the stone blocks were darker than the stone itself. Indeed, this was corroborated by the statistical analysis, which showed significant differences for all organisms considered. In terms of the a* value (which is related to changes in redness-greenness), it was possible to observe that, in general, this value decreased for the majority of the blocks, indicating a greener biofilm, except for blocks
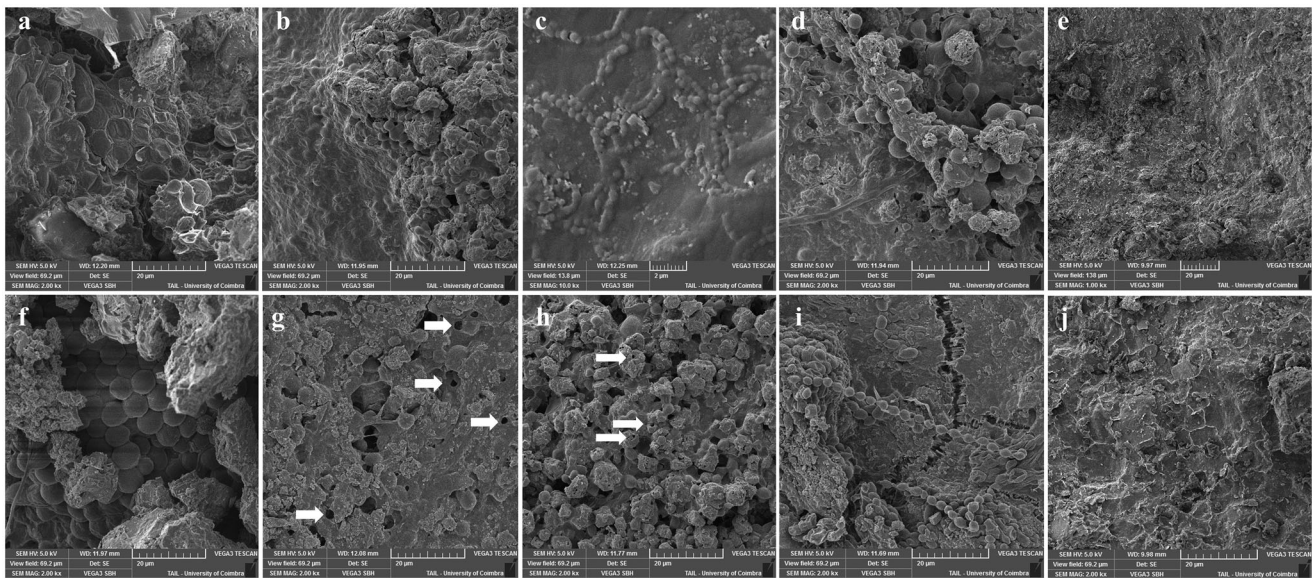
Figure 4. SEM analysis regarding phototrophic colonization on dolomitic limestone blocks after three months of incubation. Results showing: some examples regarding muciliginous matrix observed in stone blocks inoculated with *Heterochlamydomonas inaequalis* (a), *Pseudochloris wilhelmii* (b) and *Myxacorys almedinensis* (c); examples of endolithic colonization by the multispecies culture (d) and *Bracteacoccus* sp. (f); examples of the presence of small cavities (white arrows) on stone blocks inoculated with the multispecies culture (g) and *P. wilhelmii* (h); notable epilithic stone colonization by *Parakomarekiella sesnandensis* (i). Control blocks (uncolonized) (e, j).

inoculated with *P. monallantoides*, *Myxacorys almedinensis*, and the multispecies culture, where a slight increase was observed. However, no statistical differences were found for these species. In terms of the b* value (which indicates changes in yellowness-blueness), it was possible to observe an increase in this value for all stone blocks, with exception to *Bracteacoccus* sp. and *P. sesnandensis*, where a decrease could be verified. The increase in the b* value was more pronounced in stone blocks inoculated with *Heterochlamydomonas inaequalis*, *P. monallantoides*, and *M. almedinensis*, indicating a more yellowish color. This can also be corroborated by the statistical analysis, which showed significant differences for these species. In the overall, this could help explain the change from a greenness to yellowish color in the stone blocks inoculated with *M. almedinensis* (higher a* and higher b* value), as well as the less intense green color, and less developed biofilm, observed for stone blocks inoculated with *P. monallantoides* (higher a* and higher b* values), and the multispecies culture (higher a* and higher b* values). Similarly, it also explained the intense green color in the stone blocks inoculated with *Bracteacoccus* sp. (lower a* and lower b* values). Taking this information into account, it was possible to verify that the b* value increased with the biofilm development in these yellow dolomitic limestones, a peculiarity also observed when considering phototrophic colonization on white and red stone surfaces (De Muynck et al. 2009; Gambino et al. 2019; Prieto et al. 2005; Sanmartín et al. 2012). The green to yellowish biofilm color change could also be explained by the adaptation of the inoculated strains to the new conditions (ie their transference from growing on liquid BG_{11} medium in a test tube to establishing themselves on a stone surface), or due to a chromatic adaptation, which can have a genotypic or phenotypic basis (Miller et al. 2010b). For example, when in nitrogen depletion, cyanobacteria can

change their color to yellow-brown, due to a reduction in chlorophyll and phycocyanin, and an increase in the carotenoids content (Macedo et al. 2009; Miller et al. 2010b). In addition, environmental factors and ecological stages, such as light intensity, temperature, and cells age can also play a part in these chromatic changes (Alakomi et al. 2004; Bartolini et al. 2004; Miller et al. 2010b).

In stone blocks inoculated with *P. sesnandensis* and *J. aeroterrestrica*, some white spots denoting fungal and bacterial contamination, respectively, were verified. It is important to refer that the fungal contaminations were attributed to the presence of the genus *Acremonium* (Ascomycota), which could be explained by the fact that this fungus was isolated from the Old Cathedral of Coimbra, in a biofilm sample where *P. sesnandensis* was also present (see Soares et al. 2019a; Trovão et al. 2019). When performing periodic observations and subculturing of the phototrophic strains used in this study, small hyphae filaments of this fungus were sometimes observed in the test tubes that contain *P. sesnandensis*, which may have been accidentally transferred to the stone surfaces upon inoculation. Nonetheless, these results are in accordance with Miller et al. (2008), who similarly observed the presence of fungal contaminations on stone surfaces inoculated with phototrophic biofilms after three months of incubation. On the other hand, the white colonies in stone blocks inoculated with *J. aeroterrestrica* are due to a bacterial contamination (*Hydrocarboniphaga* sp.). In this case, ever since this species was isolated from the Old Cathedral of Coimbra, a white halo on the test tubes has always been present, and successive attempts to decontaminate this strain have failed. The presence of such microorganisms in microalgae and cyanobacterial cultures are usually due to co-isolation, and are believed to be distributed in the culture medium or embedded in the mucilage produced by the algae (Amaral et al. 2013). Nonetheless, this reinforces that phototrophic organisms can
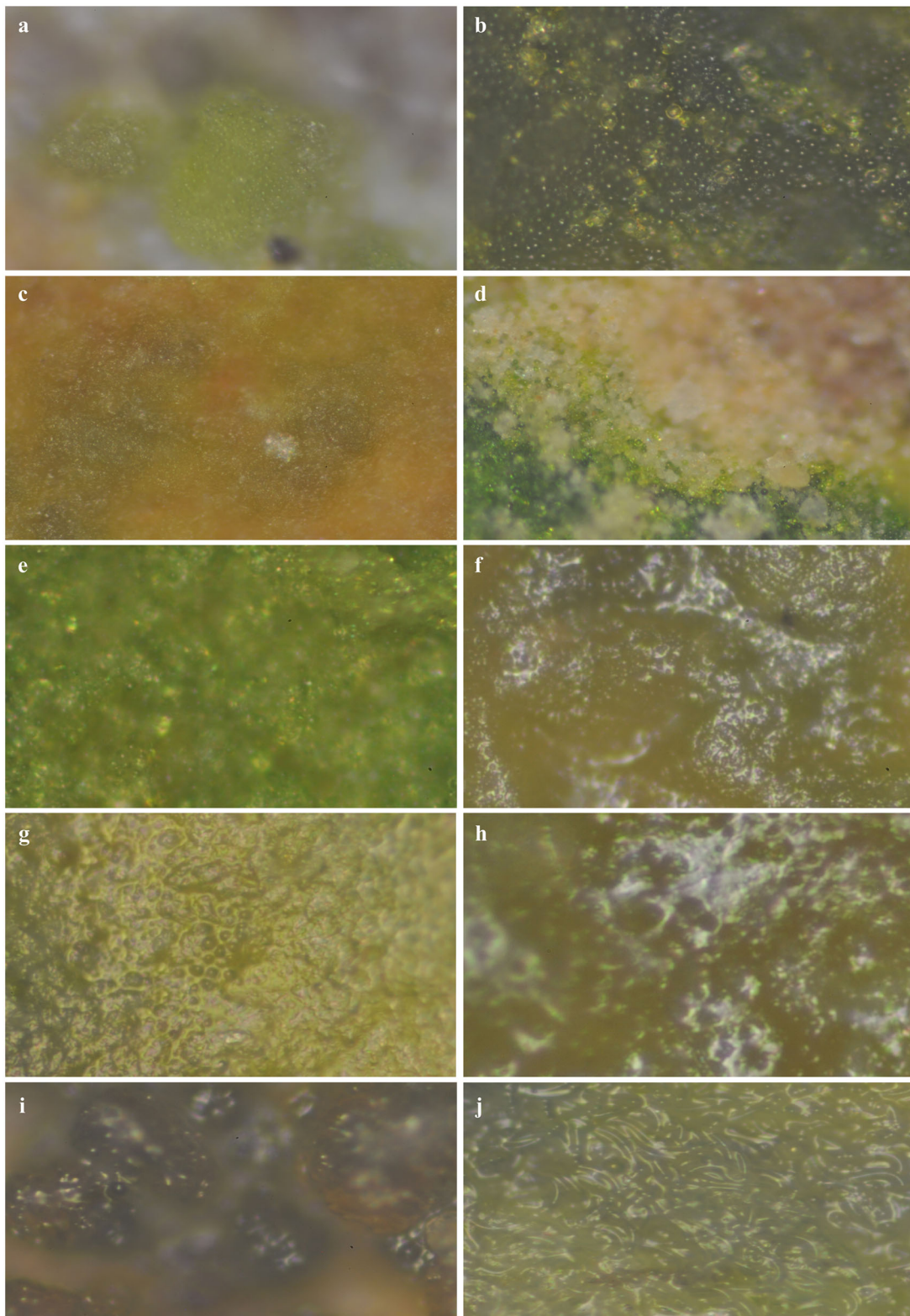
Figure 5. Stereomicroscopy images showing dolomitic limestone blocks inoculated with: *Polulichloris henanensis* (a); *Heterochlamydomonas inaequalis* (b); *Pseudostichococcus monallantoides* (c); *Bracteacoccus* sp. (d); *Acutodesmus bajacalifornicus* (e); *Pseudochloris wilhelmii* (f); *Jenufa aeroterrestrica* (g); *Myxacorys almedinensis* (h); *Parakomarekiella sesnandensis* (i); multispecies culture (j). Images in (f), (g), (h) and (j), show a mucilaginous matrix covering the surface of the stone blocks. Amplification = 20×.

provide a supply of organic nutrients for heterotrophic micro-organisms, namely, fungi and bacteria (Crispim and Gaylarde 2005; Miller et al. 2008; Saiz-Jimenez et al. 1995; Warscheid and Braams 2000).

To the best of our knowledge, apart from the green microalga *Bracteacoccus* sp., the remaining isolates of this current work have never been used in bioreceptivity and colonization studies. Vázquez-Nion et al. (2016), collected

Bracteacoccus sp. and *B. minor* from natural biofilms developed on granitic historic buildings in Santiago de Compostela, Spain, and used them in bioreceptivity studies (Vázquez-Nion et al. 2017, 2018). In this current study, although *Bracteacoccus* sp. was the species with the most developed biofilm of the experiment, it was not able to colonize a very extensive surface area. This is very interesting, as the multispecies culture was the one that showed the highest colonization area, despite having a less developed biofilm. Once again, it is hypothesized that this could be due to competition among all species, which could make individual isolates grow in less occupied areas in the surface of the stone when in the presence of other species. These results differ from those of Vázquez-Nion et al. (2017), who showed that a monospecies culture (in this case, a culture composed solely by the green microalga *B. minor*) was less adaptable to a granite substratum. However, it is unclear if these differences occur due to the type of stone substrate and their inherent characteristics, or if these differences are related to the microorganisms themselves, or other factors. Nonetheless, the results gathered in this current study demonstrated that monospecies cultures can be adaptable to dolomitic limestone substrates and that they are capable of biofilm development and stone alterations in these lithotypes. In addition, SEM results showed that some species used in this study, namely, *P. monallantoides*, *Bracteacoccus* sp., and *J. aeroterrestrica*, were able to perform endolithic colonization, which can contribute to the process of stone biodeterioration. Although this type of colonization is considered a survival strategy for when conditions on stone surfaces are adverse (Saiz-Jimenez et al. 1990; Walker et al. 2005), endolithic organisms can cause disruption of stone structures through physico-chemical processes, leading to stone deterioration (Gaylarde et al. 2020; Macedo et al. 2009; Piñar and Sterflinger 2009; Zhang et al. 2019). Besides endolithic colonization, phototrophic organisms are also capable of causing stone alterations by their epilithic colonization. This can be due to the production of biofilms of many colors, which give stone monuments an unpleasant appearance (Macedo et al. 2009), or due to carbonate dissolution and/or acid production caused by epilithic algal overgrowths (Bachman 1915; Ortega-Calvo et al. 1995; Viles 1987). The results obtained showed that all species used in this study have the capacity to cause esthetic alterations to the stone blocks, as a result of their ability to form green biofilms. The results obtained through SEM and stereomicroscopy showed an apparent mucilaginous matrix covering the surface of the stone in blocks inoculated with *P. wilhelmii*, *J. aeroterrestrica*, *H. inaequalis*, *M. almedinensis,* and the multispecies culture. The presence of a similar extracellular polymeric substances (EPS) matrix has also been reported in the work of Santo et al. (2021), who observed a well-developed biofilm covering a marble surface, contributing to grain detachment and strong adhesion. It is long known that the presence of phototrophic microorganisms on external stone surfaces is associated with unpleasant esthetic alterations, biofilm formation, organic and inorganic acid secretions, pore size alterations, and weathering (Crispim and Gaylarde 2005; Dakal and Cameotra 2012; Gaylarde and Morton 1999).

The presence of small cavities verified on stone blocks inoculated with *P. wilhelmii* and the multispecies culture resembled those observed by Hoppert et al. (2004), when green microalgae were present in a stone sample. The authors wrote that the cavities were caused by active substrate dissolution. This hypothesis is not excluded, but further studies will be necessary in order to make sure if the small cavities observed in this current work are in fact due to stone dissolution induced by the presence of the phototrophic organisms. Anyways, these small cavities were not observed in the control stone blocks. Nonetheless, biodeteriorating patterns such as cracks, micropitting, and biogenic mineral deposition have been previously associated with the presence of microalgae on stone monuments (Miller et al. 2013; Sarró et al. 2006). Although these types of biodeterioration can cause harm to stone monuments, the question whether biofilms play a biodeteriorative or a bioprotective role is still open to debate (eg, Liu et al. 2022; Pinna 2014). Biofilms can still protect stone monuments from the direct impact of rain and wind, helping to regulate thermal and moisture changes on the surface of the stones. Thus, as stone decay can also be caused by natural weathering, future studies should involve not only the composition and metabolic activities of the microbial communities but also the effects caused by abiotic factors (see Liu et al. 2022 for more detail regarding this topic).

Bioreceptivity and colonization studies are considered crucial in the field of cultural heritage, as they help to understand the susceptibility of a given stone material to microbial colonization and, consequently, to its biodeterioration (Guillitte 1995). The results gathered in this work showed that, although bioreceptivity and colonization studies employing multispecies cultures can better mimic what happens in nature, single species cultures can also play an important part in stone bioreceptivity and biodeterioration.

Acknowledgements

Disclosure statement

Funding

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