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Characterizing the microbial colonization of a dolostone quarry: implications for stone biodeterioration and response to biocide treatments

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Abstract

This study examines the microbial colonization of three fronts of an abandoned dolostone quarry (Redueña, Madrid, Spain) exposed to atmospheric conditions for different time periods since Roman times to the present. Through scanning electron microscopy in backscattered electron mode (SEM-BSE), endolithic colonization was predominantly detected in the most recently exposed front, while in the longer exposed quarry fronts, epilithic forms of growth were most often observed. These observations were confirmed by DGGE analysis. Based on the distribution pattern of microbial colonization in the different quarry fronts, we then established a sequence of colonization events that took place over this long time frame. Bioalteration processes related to this sequential colonization were also identified. Characterizing these sequential processes can be useful for interpreting biodeterioration processes in historic dolostone monuments, especially those affecting constructions in the area of the Redueña stone quarry. In a second experimental stage, different biocide treatments were tested on this quarry rock to find the best way to avoid the microbial colonization effects identified. Through combined SEM-BSE/DGGE analysis, the efficacy of several biocides against the microorganisms inhabiting the dolostones was assessed after 4 and 16 months treatment. In general, all treatments were effective at reducing around 80% of the lichen cover, although effects on endolithic lithobiontic communities were dependant on how well the rock surface had been mechanically cleaned prior to treatment and gradually disappeared over time.

Keywords: biocides, biodeterioration, conservation, dolostone, fungi, lichen, lithobionts, quarry, sequence of microbial colonization.

1

2 **Introduction**

3

4 The term biodeterioration refers to any decay process that affects monuments as the
5 result of biological colonization [4, 38, 46]. However, microorganisms are not the only
6 agents that cause the decay of monuments. Many environmental factors may
7 synergistically or antagonistically contribute to the deteriorating actions of
8 microorganisms [38]. For effective preventive measures to be designed, biodeterioration
9 processes need to be accurately identified [5, 8, 10, 12]. However, owing to their artistic
10 and historic value, monument stones cannot be extensively sampled for this diagnosis.
11 Thus, the mechanisms of bioalteration of different types of stone in natural
12 environments need to be determined and used to diagnosis biodeterioration and adopt
13 appropriate control strategies [11, 31, 38]. Firstly considered, should be treatments
14 aimed at destroying favorable conditions for the growth of the most damaging
15 microorganisms [10, 43]. However when this is insufficient, specific treatments have to
16 be used to prevent as well as eliminate biological colonization. According to Ascaso et
17 al. [5], these treatments should be ideally assessed *in situ*, without extracting the
18 microorganisms from their microhabitat. **The viability of cells after treatment can be
19 examined using different methodologies. Microscopy techniques provide valuable
20 information on the integrity of cell membranes and ultrastructural changes, but
21 complementary techniques such as chlorophyll-a fluorescence detection can reveal
22 changes in the physiological state of free-living or lichenized phototrophic
23 microorganisms [44, 45].**

24 Stone quarries are an ideal natural environment to carry out this type of study
25 since there is a non-limited supply of sample material and the information obtained is

1 directly transferable to any nearby monuments built out of the same rock [10, 15, 19]. A
2 further benefit of this type of sampling site is that the mineral surfaces of exploited
3 natural outcrops are of known age, such that it is easy to calculate time frames for the
4 development of biofilms and their weathering rates [6, 10].

5 Several studies have been performed in old quarries of different rock types (see
6 references in [15]). For example, some of the most recently reported applications of
7 such studies have been the induction of the growth of biofilms in a quartz quarry to
8 correct the visual impacts of past mining activities [36] and bacterially induced
9 mineralization of quarry stone to protect and consolidate new stone pieces to replace the
10 damaged stone of a historic building [29].

11 The Madrid region has several ancient stone quarries and much of the region's
12 architectural and monumental heritage was built from stone materials extracted from
13 these sites [24]. The Redueña site is a Cretaceous dolostone quarry in the north of
14 Madrid, whose characteristics of workability, availability and proximity to urban areas
15 made its stone one of the most used materials for the construction of historic
16 monuments in the region from Roman times until the mid-twentieth century [32].
17 Although many of these dolostone monuments still stand today, signs of deterioration
18 are visually apparent.

19 The aim of this study was to characterize the types of lichen and microbial
20 colonization of different fronts of the Redueña quarry exploited at different times in
21 history using an integrated microscopy and molecular biology approach. The
22 information obtained will be used to identify biodeterioration processes related to these
23 types of colonization in historic monuments built out of this stone. In addition, we
24 evaluated the *in situ* effects of different biocide treatments on the lithobiontic
25 communities inhabiting the microhabitats of the Redueña quarry to design an
26 experimental assay for future use on monument stones.

27

28 **Methods**

29 *Sampling*

1 Pieces of dolostone were taken from the Redueña quarry. This natural outcrop forms
2 part of a discontinuous narrow band of Upper Cretaceous dolostone running NE-SE in
3 the Spanish Central System (917 m above sea level; N 40° 47.66'; O 3° 33.25'). From
4 Roman times to the mid-twentieth century, dolostone from the Redueña quarry has been
5 intermittently used to construct monuments of different architectural styles in the
6 region. Today the quarry has three different fronts that were exploited in different time
7 periods. The increasing dimensions of these fronts and ashlar over the centuries reflect
8 improvements in the tools used to extract the stone facings typical of the time and new
9 forms of transport [32].

- 10 ■ Quarry Front I (QF-I): This is the largest front, which was mainly exploited to
11 reconstruct most of the existing dolostone historic monuments in the 1940s.
12 Materials were extracted from three sites ordered in time. The base of this front was
13 the most recently abandoned.
- 14 ■ Quarry Front II (QF-II): The rocks of this front were intermittently extracted in the
15 XIV-XVI centuries.
- 16 ■ Quarry Front III (QF-III): This is the oldest and smallest front (Fig. 1A), which
17 could correspond to the start of dolostone extraction activities in the area. The
18 earliest mining activities have been dated as Roman based on evidence of the
19 rudimentary methods used to extract the stone [32].

20 To examine microbial colonization, representative samples were collected from
21 each of the fronts (I, II, III) of the Redueña quarry in October 2006. In all cases, rock
22 specimens were taken from a vertical south-facing wall, since throughout history it was
23 mainly this southern aspect of the three quarry fronts that was exploited. The same
24 samples were used for scanning electron microscopy (SEM-BSE), isolating fungi and
25 DGGE analysis.

26 In addition, samples were collected from historic monuments constructed using
27 Redueña dolostones as dimension stone to compare with the quarry stone including the
28 Roman Bridge (Talamanca del Jarama, II century), and the Inmaculada Concepción
29 church (Soto del Real, XVI-XVIII centuries).

31 *Biocide experiments*

32 The effectiveness of different biocide treatments was tested in the oldest quarry front
33 (Q-III) since this one was the most extensively colonized. The experiment was

1 conducted in four 25 x 25 cm grid plots on a vertical surface (C: control; T1: treatment
2 1; T2: treatment 2 and T3: treatment 3) (Fig. 1A). The biocide treatment was
3 administered according to the manufacturer's instructions. This consisted of
4 mechanically removing the biological cover using a sponge followed by application of
5 the chemical compounds. Three commercial biocides provided by THOR were applied
6 in different combinations and concentrations using a paintbrush: ACTICIDE® LV706
7 (10% in distilled water), ACTICIDE® CF (1.5% in water-repellent resin) and
8 ACTICIDE® IOG (0.5% in water-repellent resin). The experiment was performed on
9 three consecutive days in July 2007. The treatment regimens and compositions are
10 shown in Table 1. Samples were collected 4 and 16 months after applying the biocides
11 and their efficacy assessed in terms of the reduction in lichen cover (%), changes in
12 chromatic variables (L*, a*, b*, C*, IB, IA) and analysis of the lithobiontic
13 communities by SEM-BSE and DGGE.

14

15 *Mercury intrusion porosimetry (MIP)*

16 The porosity and pore size distribution of the Redueña dolostone were determined using
17 a Micromeritics Autopore IV 9520 mercury intrusion porosimeter. The limit between
18 micro- and macroporosity was set at a pore diameter of 5 µm.

19

20 *Polarized optical microscopy (POM)*

21 Thin sections of samples from the three quarry fronts were petrographically
22 characterized using an Olympus BX 51 microscope connected to an Olympus DP 12
23 6V/2.5Å) digital camera. The software used was Olympus DP-Soft (version 3.2).

24

25 *Epilithic colonization cover*

26 Epilithic colonization was estimated in each experimental plot by measuring percentage
27 cover in 12 randomly selected zones of smaller size (4 x 4 cm) through digital image
28 analysis using the UTHSCSA Image Tool 2.0 (University of Texas Health Science
29 Center in San Antonio, USA). Cover values for each treatment at the two time points (4-
30 and 16- months after application) were compared by two-way analysis of variance,
31 including biocide treatment and time as categorical predictors. Tukey's HSD post-hoc
32 test was used to identify differences among treatments. All statistical tests were
33 performed using STATISTICA 6.0 (Tulsa, OK, USA).

1

2 *Chromatic characterization of the experimental plots*

3 Colonization cover was also determined colorimetrically at the 16-month time point.
4 The color of each experimental plot was measured by reflectance using a MINOLTA
5 CM 700d spectrophotometer and analyzed by COLOR DATA SPECTRAMAGIC™
6 NX CM-S100W (Minolta) software. Results are expressed as the mean of 20 direct
7 readings following two diagonals in each of the experimental plots. The chromatic
8 variables determined defining the color of a material were: L* (lightness), a* and b*
9 (chromaticity coordinates indicating red (+a*), green (-a*), yellow (+b*) and blue (-b*)
10 directions), C* (chroma or saturation defined by the equation $C^* = \sqrt{(a^*)^2 + (b^*)^2}$), IB
11 (whiteness index) and IA (yellowish index) [16]. The L*/C* diagram represented here
12 was the most appropriate to determine differences in color between the untreated and
13 treated plots.

14

15 *Scanning electron microscopy with back-scattered electron imaging (SEM-BSE)*

16 Fragments of dolostone from the different quarry fronts, historic monuments and
17 biocide-assay plots were prepared for SEM-BSE according to a technique [48] described
18 elsewhere with some modifications. Briefly, hand-cut pieces of rock were fixed in
19 glutaraldehyde (3.25% in 0.05 M cacodilate buffer) and then in osmium tetroxide
20 solution (1% in 0.025 M cacodilate buffer), dehydrated in a graded series of ethanol
21 solutions and embedded in LR-White resin. Samples finely polished and carbon-coated
22 were observed in a Zeiss DMS 960 SEM equipped with a four-diode, semiconductor
23 BSE detector and an ISIS Link EDS microanalytical system.

24

25 *Isolation of fungal strains*

26 Free-living fungi were isolated from deep areas of recently fractured rock samples.
27 Small fragments of each sample were incubated on 1.5% malt extract agar (MEA) at
28 room temperature for 3-4 weeks. Isolates were purified in two or three steps by transfer
29 to fresh medium.

30

31 *DNA extraction from rock samples and fungal cultures*

32 To characterize microbial colonization, DNA was extracted from epilithic and
33 endolithic areas (~ 0.5-1cm below the rock surface) of the rock samples. For DNA
34 extraction, the Power Soil DNA Isolation kit (MoBio Laboratories Inc, Solana Beach,

1 CA, USA) was used according to the manufacturer's instructions. The extraction
2 process was started by resuspending 250 mg of sample material in lysis solution.

3 To identify the fungal strains isolated, total DNA was extracted from different mycelia
4 according to a modified CTAB method [9].

5 6 *PCR amplification and sequencing*

7 The ITS regions of the rRNA gene were used for PCR-amplification. For the DGGE
8 analysis of environmental samples, the primer pair used was ITS4GC/ITS3 [47] and to
9 obtain the sequences of the isolated fungal strains the ITS1F/ITS4 pair [22, 47].

10 PCRs were carried out in a 25 µl final volume using Illustra™ puReTaq Ready To-Go
11 PCR Beads (GE Healthcare) containing 0.1 U/µ of puReTaq DNA polymerase in Tris-
12 HCl (pH 9.0), 50 mM KCl and 1.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each
13 primer and ca 10-50 ng of genomic DNA. The thermocycling program was: 5 min
14 denaturation at 94°C, followed by 30 cycles at 95°C for 30s, 52°C for 1 min and 72°C
15 for 1 min 30s. The final extension step was 10 min at 72°C or 30 min at 72°C for the
16 DGGE samples.

17 The PCR products from the fungal cultures and DGGE bands were cleaned on a
18 QIAGEN quick spin column (QIAGEN, Valencia CA). Both complementary strands
19 were sequenced separately at the SECUGEN sequencing company (S.L. Madrid,
20 Spain).

21 22 *Denaturing gradient gel electrophoresis (DGGE)*

23 DGGE was performed using a D-Code Universal Mutation Detection System (Bio-rad).
24 Samples (22 µl) were loaded on 6% acrylamide/bisacrylamide (37.5:1) gels with a
25 denaturing gradient of 30-60% (where 100% denaturant is 7 M urea and 40%
26 formamide). The gels were run at 70 V for 16 h at 60°C. After staining with ethidium
27 bromide 10 mg/ml for 10 min, the gels were rinsed in distilled water prior to viewing
28 and capture in a UV transilluminator.

29 Well-defined DGGE bands were excised using a scalpel blade and incubated at 4°C
30 overnight in sterile distilled water and then reamplified using the same primers as for
31 PCR but without the GC-clamp. The resultant PCR products were purified using a
32 QIAGEN quick spin column (Qiagen) and sequenced at the SECUGEN company (S.L.
33 Madrid, Spain)

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Results

Mineralogical study

The samples from the three quarry fronts showed the same petrographic features confirming they all belonged to the same Cretaceous dolostone outcrop. The Redueña stone is a dolostone with a massive internal structure comprised mainly of rhombic, idiomorphic dolomite crystals (MgCa_2CO_3) [18]. These clean and unaltered crystals form dense crystalline masses of fairly uniform size (70-80 μm). The presence of high porosity ($\approx 20\%$) should be highlighted. Main porosity is intercrystalline of regular morphology and small size (100-150 μm) (Fig. 1B), although a larger and more irregular intercrystalline porosity (0.5-1 mm) may occasionally appear infilled with crystals of calcite cement. A further type of porosity appears close to the surface and is attributable to surface weathering. These cracks (fissure porosity) (Fig. 1C) run parallel to the surface and are only a few millimeters deep. The pore size distribution plot for the Redueña dolostones determined by mercury intrusion porosimetry indicates that 17.9% of the total porosimetry is in the range of 400-0.01 μm of pore size(Fig. 1D).

Microbial colonization

Microbial colonization in the three quarry fronts was macroscopically and microscopically assessed to compare epilithic and endolithic communities in terms of their cover, spatial occurrence and weathering actions.

Microbial colonization of the quarry front exploited in the 1940s (QF-I).

This quarry front was the most recently exposed of the three examined. Because of its large size, we sampled three sites following an exposure time gradient. At the macroscopic scale, epilithic lichen thalli were practically absent on the rock surface at the base of the front (Fig. 2A) but increased in frequency towards the top (Fig. 2B). These observations were confirmed by SEM-BSE. Thus, at the base of this front, extensive endolithic fungal colonization was predominantly observed (Fig. 2C). The scarce epilithic growth was associated with fragmentation and detachment of dolomite crystals (arrowheads in Fig. 2C). In contrast, at the top of this front or site exploited longest ago, the rock surface was colonized by small lichen thalli, some of which

1 showed mineral particles in their interior (arrowheads in Fig. 2D). At this site, it was
2 also possible to detect fungal hyphae inside the rock (empty arrow in Fig. 2D),
3 approximately 200 μm below the surface. Occasionally, besides colonizing the
4 intercrystalline porosity of the dolomite crystals, these fungi also penetrated the crystals.

5
6 *Microbial colonization of the quarry front exploited between the XIV-XVI centuries*
7 *(QF-II).*

8 In this area, epilithic colonization was more extensive than in QF-I (Fig. 3A), with
9 an increase in lichen thallus size observed (Fig. 3B). Microdivided mineral crystals of
10 different size were trapped among the mycobiont hyphae, which showed a capacity for
11 intense mechanical weathering. In the attachment area, numerous fungal hyphae
12 emitting an intense BSE signal were closely associated with the lithic substrate (black
13 arrow in Fig. 3B). These hyphae generated an extensive network of colonized fissures
14 and cracks inside the rock (arrowheads in Fig 3B). Clusters of algal cells and fungal
15 hyphae were also frequently observed in deep areas of the dolostone, also forming an
16 extensive network inside the rock through the spaces between the dolostone crystals
17 (arrows in Fig. 3C).

18
19 *Microbial colonization of the quarry front exploited in Roman times (QF-III).*

20 At this sampling site, abundant epilithic lichen colonization was both macroscopically
21 and microscopically observed (Fig. 4A-B). *Verrucaria nigrescens* Pers. was found to
22 colonize extensive areas of the rock. The weathering capacity of this lichen was
23 reflected by the presence of several mineral fragments completely embedded in its
24 medulla (asterisk in Fig. 4B), possibly as the result of its mechanical actions. Hyphae
25 were also seen to colonize deep areas of the dolostone (arrows in Fig. 4B), mainly
26 through its intercrystalline porosity (arrows Fig. 4C) and intracrystalline pores
27 (arrowheads).

28
29 ***Fungal isolation***

30 Ten fungal strains were isolated in culture medium (MEA 1.5 %) and identified using
31 the ITS rRNA region as a genetic marker (Table 2). Most of the isolates were identified
32 as ascomycetes and basidiomycetes. All isolates came from quarry fronts QF-I and QF-
33 II. No fungal strains could be isolated from the oldest quarry front (QF-III).

34

1 *DGGE profiles*

2 The structure of the epilithic and endolithic free-living and lichenized fungal
3 communities was analyzed by DGGE (Fig. 5). The DGGE profiles of rRNA isolated
4 from epilithic zones tended to show a number of bands that increased with time of
5 exposure to the atmospheric conditions. Some bands of the same electrophoretic
6 mobility were shared by epilithic growths from the three different fronts (1 in Fig. 5)
7 while others only appeared in one of the fronts. In addition, some of the bands were
8 detected in both epilithic and endolithic locations (2 in Fig. 5). The number of bands in
9 all the endolithic profiles was low and decreased with quarry front exposure time.
10 Notably, some of the endolithic forms appeared only in the fronts recently exploited. In
11 Ic, II and III, endolithic profiles were restricted to a single phylotype, not detected at
12 epilithic locations (3 in Fig. 5).

13

14 *Analysis of monument rock*

15 Patterns of microbial colonization and weathering detected in monument stones (Fig. 6),
16 were similar to those observed in the rock quarry. In areas mainly colonized by lichen
17 thalli, intense alteration of the rock surface was observed and this was mainly due to the
18 mechanical actions exerted (Fig 6A). This mechanical action was especially aggressive
19 in the attachment area, where numerous fragments of dolomite crystals appeared
20 between the mycobiont hyphae (Fig. 6B) As observed in the natural dolostone, these
21 bioalteration processes were not only associated with epilithic lichens but also with
22 deep extensive colonization by endolithic mycobiont hyphae (empty arrow in Fig. 6C).
23 In addition to lichen colonization, it was possible to observe the microbial colonization
24 of both epilithic and endolithic locations. Thus, the hyphae of free-living fungi were
25 frequently detected in areas below lichen thalli (arrows in Fig. 6D), but were also
26 commonly observed in areas where epilithic lichens were absent (Fig. 6E), resembling
27 the early stages of microbial colonization of the Redueña dolostones described above.
28 Bacteria were also observed in the proximity of mycobiont hyphae penetrating the
29 substrate (black arrows in Fig. 6C). Finally, algae sometimes appeared both on the rock
30 surface and deep within **some of these** monument dolostones (Fig. 6F).

31

32 *In situ biocide efficacy against the lithobiontic community*

33 The efficacy of the different biocide treatments (T1, T2 and T3) applied to the oldest
34 quarry front (QF-III) was assessed in the short- (4 months) and long-term (16 months).

1 After 4 months of biocide treatment, a highly significant reduction ($F= 373$; $p<0.0001$)
2 in lichen cover approximating 80% of the total cover was recorded in the three
3 experimental plots compared to the control (Fig. 7A-I). However, no significant
4 ($F=1.60$; $p=0.194$) differences between biocide treatments were detected. After 16
5 months of treatment, these effects persisted though a marginally significant ($F=8.20$;
6 $p=0.06$) increase in epilithic cover was detected for all three biocide treatments.

7 The chromatic parameters (L^* , lightness and C^* , chroma) recorded at 16 months
8 confirmed these observations. Hence, L^*/C^* values indicated lightness ($L^*>65$)
9 increases induced by all three biocide treatments compared to the control plot which
10 showed values close to those for a gray color ($L^*<10$ and $C^*\sim 55$) (Fig. 7A-II). Both
11 techniques provided similar information and we plan to conduct further color
12 measurements on experimental plots in the future because of benefits such as speed,
13 repetitive sampling and easy monitoring of the effectiveness of biocide treatments [36].

14 Biocide effects on the lithobiontic community were determined at the cellular level
15 through SEM-BSE. This quarry front showed abundant epilithic lichen colonization
16 (Fig. 4), which was not observed after the treatments (Fig. 7B-F). However, treatment
17 effects on all microbial colonizers were not always the same. In areas where there were
18 no remains of lichen thalli on the rock surface after biocide treatment endolithic fungal
19 hyphae and algal cells lost their typical morphology, especially in places close to the
20 rock surface (Fig. 7B and inset). This effect disappeared at greater depths where we
21 observed unharmed endolithic microbial cells. When there were large amounts of lichen
22 thalli remains, it was possible to observe an interface of living and non-living cells (see
23 above and below –white arrow and black arrow– the line in Fig. 7C). After 4 months of
24 treatment, no differences among samples subjected to the different biocides were
25 observed.

26 In the longer term (16 months), most of the biocide effects observed at 4 months were
27 also detected (Fig. 7D, E, F). However, of note was the detection in some endolithic
28 areas of extensive fungal colonization inhabiting spaces between the dolomite crystals
29 (Fig. 7D and inset), absent in the untreated samples. It should also be noted that in this
30 area, most of the calcite cement in intercrystalline spaces had disappeared. In other
31 areas, abundant fungal hyphae were detected not only at endolithic locations (Fig. 7E),
32 but also at specific points on the rock surface (Fig. 7F). Our DGGE analysis of the
33 fungal endolithic community after 16 months of treatment revealed some differences in
34 profiles between the three biocide treatments and control (Fig 8). First, the control

1 profile contained one band not present in the other profiles (arrowhead), indicating an
2 endolithic form sensitive to the biocide treatments. Second, there were bands common
3 to all profiles (empty arrow), meaning the resistance of some phylotypes to treatment.
4 And finally, the recolonization of endolithic positions by new fungal forms was
5 revealed by the appearance of new bands after the T2 and T3 treatments (black arrows).
6 This observation was **complemented** by sequencing some of these DGGE bands, which
7 indicated the presence of some lichenized fungal forms after 16 months of biocide
8 treatment.

10 **Discussion**

11 Through *in situ* electron microscopy examination of the microbial colonization of an
12 abandoned dolostone quarry (Redueña), we were able to assess the extent of
13 colonization of the different quarry fronts (QF-I, II, III) corresponding to different time
14 periods. Free-living and lichenized fungi were the main inhabitants of these dolostones,
15 and these occupied different positions and showed different types of colonization. Each
16 type seemed to correspond to different stages of colonization of the dolostones. Thus, it
17 was possible to establish a sequence of fungal colonization. Colonization begins at
18 endolithic sites and epilithic locations are colonized at a later stage. Thus, endolithic
19 fungi were pioneers and epilithic lichens appeared as the predominant forms later on. It
20 is also worth mentioning that endolithic communities showed differences in
21 composition when the rock surface was colonized by lichen thalli, a finding supported
22 by DGGE and culture techniques. Accordingly, when lichens were present on the rock
23 surface, a lower diversity of free-living fungi was detected, which could indicate some
24 degree of competition.

25 The fungal pioneer endolithic colonization observed here could have arisen from
26 spores or vegetative propagules transported by wind or water, but we cannot rule out the
27 possible spread of endolithic growth from nearby areas [7]. The **development** of
28 lithobiontic communities is influenced by abiotic factors, such as the physico-chemical
29 properties of the lithic substrate, by interrelations between different community
30 members and the substrate itself [13], **but also by the specific mode of reproduction and**
31 **growth rate of each type of microorganism. In effect, ascospores of lichenized fungi in**
32 **the natural environment need to invest more time to find a compatible photosynthetic**
33 **partner and undertake morphogenesis processes relative to the time taken for the**

1 reproduction and growth of non-lichenized fungi [28, 34]. The prior presence and
2 activity of endolithic microorganisms could help the lichen symbiosis to settle in later
3 stages of colonization [13, 27].

4 Changes in the endolithic community, such as the loss of some endolithic fungal
5 forms in more developed communities, could also be related to the effects of the lichen
6 thalli on the substrate. Indeed, it is well-known that lichens interact geochemically and
7 geophysically with the lithic substrate leading to the formation of different
8 microhabitats and chemical microenvironments beneath them [3, 6, 7, 13]. Other biotic
9 interactions involved in the succession of saxicolous lichen communities have been
10 described [1, 30, 35, 49] including competition for limiting resources such as space,
11 nutrients and light [26]. The fungal communities examined here may have also been
12 shaped by competition between lichens and free-living endolithic fungi [7].

13 The chemical composition and intrinsic properties of the lithic substrate could also
14 affect the intensity and type of microbial colonization present [23, 25]. Indeed, the
15 textural features of Redueña dolostone could be one of the main determinants of the
16 type of colonization observed in this study. The extent and type of porosity of
17 dolostones have been reported to condition fungal penetration pathways along their
18 intrinsic discontinuities [8]. Thus, the lack of intracrystalline porosity in the Redueña
19 dolostone could favor the intercrystalline penetration of fungi. This has been recently
20 described by Favero-Longo et al. [15], who stressed that differences in the modality of
21 mycobiont hyphae penetration (depth of maximum penetration and penetration pattern)
22 are basically dependent on the mineral composition and structure of the different
23 lithotypes rather than on species differences.

24 The dynamics of microbial colonization has been scarcely explored. However, a
25 microbial colonization sequence similar to that observed here was described by Hoppert
26 et al. [27] in limestone across an area of receding glaciers in the Austrian Alps. The first
27 microbial settlers were endoliths encompassing a wide variety of microorganism
28 groups. However, epilithic lichen colonization was not detected in these limestones
29 exposed to the environmental conditions for 100 years. Ascaso et al. [2] also described
30 a process of sequential colonization of carbonate rocks (dolostones and biocalcarenes)
31 in the Mola quarry (Novelda, Alicante), but the sequence differs to that observed in the
32 present study. Thus, in the abandoned area of the Novelda quarry epiliths appeared,
33 while in the limiting zone of the quarry, an non-exploited area, lichens and
34 cyanobacteria occupied both endolithic and epilithic positions. This difference in the

1 sequence of colonization could be attributed to differences in mineralogical
2 composition, textural features and petrophysical characteristics [17, 18], but also to
3 climate differences (Mediterranean in Alicante *versus* continental in Madrid). Indeed,
4 seasonal and environmental variations have often been incriminated in changes in the
5 dynamics of colonization and composition of microbial communities, as described in
6 different marble stones from both quarries and monuments [19, 20, 21].

7 We detected similar bioalteration processes in dolostone from the quarry and from
8 monuments built out of the same stone. Long-standing colonization by lichen
9 communities has meant intense alteration of the lithic substrate beneath these
10 communities. These effects appear mainly due to mechanical actions of the lichens
11 although we cannot rule out the possibility of chemical alterations. Several stages of
12 dolostone alteration were observed, similar to those described by De los Ríos et al. [11].
13 Thus, areas colonized by epilithic lichens were the most weathered and as we moved
14 away from the lichen thalli, alteration of the rock substrate diminished with depth and it
15 was possible to distinguish the other stages of alteration described by these authors.
16 However, we should not underestimate the decay of the lithic substrate in deeper areas
17 by endolithic forms under these lichens [37]. In effect, slow-growing fungi showing a
18 high similarity to *Phaeococcomyces* sp. (98.6%) and *Coniosporium* sp. (99.8%) were
19 isolated from deeper areas of the dolostones. These microcolonial fungi are common
20 inhabitants of rocks and considered very aggressive biodeterioration agents in stone
21 monuments [39, 40, 41], since their melanized pigmentation is thought to confer extra-
22 mechanical strength to the hyphae [14, 42]. All our results indicate that observations in
23 quarry stones may be translated to the situation in the historic monuments in which
24 Redueña dolostone was used as dimension stone. All this detailed information of
25 microbial colonization strategies obtained in this study, especially the presence of
26 different endolithic forms, helped us select treatments to combat the problems caused by
27 the entire lithobiontic community.

28 By *in situ* assessing the biocide treatments on quarry stone, our aim was to simulate
29 one of the strategies commonly used in the conservation of historic monuments while
30 avoiding any risks of damaging this cultural heritage. The biocide treatments *per se*
31 were efficient in that they all managed to reduce lichen cover by over 80%. However,
32 the lack of significant differences between treatments ($p=0.194$) indicates that the most
33 resolving strategies were mechanical cleaning and treatment with ACTICIDE® LV706
34 (disinfectant plus fungicide) since these were common to all the experimental plots. In

1 addition, the effectiveness of treatment was dependent on the persistence of lichen
2 remains after cleaning the rock surface indicating that our cleaning method was not
3 totally effective and should be modified in future experiments. These lichen remains act
4 as a protective layer, avoiding the penetration of the agents into deeper areas of the
5 lithic substrate and their actions on endolithic microorganisms. Irregularities in the rock
6 surface could be the main reason for the lack of effectiveness of the treatments in some
7 areas. The recolonization of dolostones observed after 16 months of biocide treatment
8 reveals a need for retreatments. Effectively, the presence of remains of dead free-living
9 and lichenized fungi and the absence of competitors could have promoted the growth of
10 free-living fungi [10, 33]. Future experiments should be aimed at mitigating the effects
11 of the factors that could have interfered with the success of the biocide treatments tested
12 here before these biocides are used on these monuments. Other measures that could be
13 tested in future trials are, for example, wetting the rock surface and applying a higher
14 biocide concentration as pretreatment before mechanical elimination of the epilithic
15 biomass [33, 44, 45].

16 Our findings also indicate that the Redueña quarry is an ideal natural laboratory for
17 analyzing lithobiotic communities and the efficacy of biocide treatments designed to
18 eliminate them. Indeed, this use of quarry stone is recommended to optimize the
19 different protocols and conditions in the *in situ* evaluation of biocide treatments [29].
20 Once the most effective method has been established, this information could be
21 extrapolated to monuments built out of the same rock. The sequence of microbial
22 colonization of natural dolostones inferred here and the *in situ* assessment of biocide
23 treatments contribute to our present understanding of the microbial ecology of
24 lithobiotic communities and also have implications for conserving our cultural
25 heritage.

26

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Figure Legends

Fig.1. **A.** General view of the quarry front in which the last mining activity dates back to Roman times (QF-III) showing the different experimental plots used to assess the efficacy of the biocide treatments (C: control; T1: disinfectant + fungicide a; T2: T1 + (fungicide a +algicide); T3: T1 + (fungicide a + fungicide b); **B.** POM micrograph under parallel polars of the Redueña dolostone revealing it is mainly composed of rhomboedric dolomite crystals and shows high intercrystalline porosity (Ip). Intercrystalline pores are sometimes filled with calcite cement; **C.** POM micrograph under parallel polars of fissure porosity (Fp) associated with the exposed surface. Note that this type porosity only affects the first 1.5 mm of the rock; **D.** Curve of the pore size distribution of the Redueña dolostone estimated by mercury intrusion porosimetry: 17.9% of the total porosity is in the range 400-0.01 μm .

Fig 2. Time-dependent succession of endolithic and epilithic fungal growth in QF-I; **A-B:** Images showing a macroscopic view of the different microbial colonization stages detected in QF-I; **C.** First stage of colonization detected in QF-I. SEM-BSE image of endolithic fungal colonization randomly distributed throughout the rock. Note the surface growth of these endolithic forms promotes disaggregation of the dolomite crystals (arrowheads) **D.** SEM-BSE image of small lichens (Li) colonizing the lithic surface in an advanced stage of colonization corresponding to the images in Fig. 2B. Note the mineral fragments embedded in some of the lichen thalli (arrowheads). Endolithic fungal colonization (empty arrow) appears beneath the lichen thalli, occupying pores between the dolomite crystals, but also penetrating the crystals

Fig 3. **A-C.** Microbial colonization in QF-II. **A-**Image showing a macroscopic view of the epilithic cover; **B.** SEM-BSE image of intermediate-size epilithic lichen (Li). The mycobiont's hyphae (arrowheads) are strongly attached to the lithic substrate provoking fragmentation of the dolomite crystals (arrows); **C.** Endolithic colonization occurring as algal and fungal associations in the intercrystalline porosity of the dolostone (arrows).

Fig 4. **A-C.** Microbial colonization in QF-III. **A.** Macroscopic view of the epilithic colonization in QF-III; **B.** SEM-BSE of large epilithic thalli (Li). Mineral fragments

(asterisk) of variable size appear embedded in the medulla of a lichen between mycobiont hyphae, indicating the lichen's weathering capacity; **C**. Mycobiont hyphae detected in deep areas dolostone substrate colonizing intercrystalline (arrows) and intracrystalline (arrowheads) pores of dolostone.

Fig 5. DGGE profile of the fungal diversity of epilithic (EP) and endolithic (EN) locations in the different quarry fronts (I, II, III). DGGE banding patterns correspond to the ITS regions of the rRNA gene (~400 pb): (1) bands common to epilithic positions in the different quarry fronts; (2) bands appearing both in epilithic positions and endolithic positions; (3) bands common to all the endolithic locations in the different quarry fronts not present in the epilithic profiles.

Fig 6. SEM-BSE images of lichen and microbial colonized areas in historic monuments built using Redueña dolostones (d); **A**. Epilithic lichen thallus (Li) provoking intense alteration of the dolostone (arrows); **B**. Fungal hyphae (arrows) surrounding dolomite crystals (d) in endolithic positions beneath a lichen thallus; **C**. Epilithic lichen thallus (Li) with extensive endolithic colonization (empty arrow) where mycobiont hyphae coexist with other microorganisms (black arrows); **D**. Lichen thallus (Li) colonizing the rock surface of dolostones from the church La Inmaculada Concepción (Soto del Real, Madrid). Note fungal hyphae emitting an intense BSE signal occurring several micrometers beneath the lichen thallus (arrows); **E**. Endolithic colonization by free-living fungi inhabiting preexisting fissures in the dolostone (arrows) in the absence of epilithic lichens. **F**. Epilithic colonization by algae (arrows) in dolostones from the Roman bridge at Talamanca del Jarama (Madrid).

Fig 7. Assessment of the efficacy of the biocide treatments on the Redueña quarry dolostone; **A-I**. Epilithic lichen cover determined in the experimental plots at 4 and 16 months post treatment. After 4 months of treatment, epilithic cover was reduced to 1/5 and no differences between treatments were observed. Different letters indicate significant differences; **A-II**. Diagram of L* (lightness)/C*(chroma) of the experimental plots measured by reflectance using a spectrophotometer. The treated plots show similar lightness values which were higher than the value recorded for the untreated plot; **B-C**: SEM-BSE images of the effects at 4 months on the lithobiontic community commonly observed in the three treatment plots; **B**. Area with no lichen remains on the rock

surface. Detailed view of the endolithic alga cells and fungal hyphae showing loss of their typical morphology; **C**. Remains of a lichen thallus. Note the interface (line) between the zone of biocide action (white arrow) and the zone of no biocide action (black arrow); **D-F**. SEM-BSE images of samples from plots after 16 months of treatment; **D**. Abundant endolithic colonization of Redueña dolostone in areas lacking lichen remains. Note that associated with this fungal endolithic colonization there is intense alteration of the internal structure of the dolostone, especially in areas with calcite cement (arrows). Detailed view of the area showing fungal hyphae with numerous lipid bodies; **E**. Area extensively colonized by endolithic fungi showing incipient epilithic recolonization (arrows); **F**. Detailed view of the newly colonized area indicated with arrows in Fig 7E.

Fig 8. DGGE analysis of the fungal endolithic colonization of the Redueña dolostone 16 months after biocide treatment. The control (C) is included for comparison. Note that some bands are common to all the profiles (empty arrow), new bands appear in some of the profiles for the different treatments (black arrow) and one of the bands only appears in the control profiles (arrowhead). (a, b, c) DGGE bands and percentage Genbank matches obtained for the control and T2 profiles: (a) *Verrucaria csernaensis* [FJ664723] 94%; (b) *Caloplaca concreticola* [EU192154] 98%; (c) *Aspicilia contorta* [EF332108] 95%.