**Differential effects of biocide treatments on saxicolous communities:
case study of the Segovia cathedral cloister (Spain)**

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ABSTRACT

This paper reports the differential elimination of surface lichen thalli and their penetrating hyphae and epilithic and endolithic cyanobacteria by two biocide treatments applied on a small scale to the cloister of the Segovia cathedral. Both chemical treatments were followed by mechanical cleaning and their effects determined by scanning electron microscopy with backscattered electron imaging (SEM-BSE). Both treatments were effective at killing epilithic lichens and cyanobacteria yet showed different effects on endolithic microbiota. The cleaning procedure involving Biotin T dissolved in water

treatment, applied to areas extensively colonized by crustose lichens, resulted in a cleaner stone surface that was practically free of surface epilithic lichen remains, but the presence of living fungal hyphae was detected in fissures of the stone under these remnants in samples examined two months after treatment. The procedure involving Biotin R dissolved in White spirit treatment left more superficial lichen remains but was better at eliminating mycobiont hyphae penetrating the stone. Endolithic cyanobacteria from zones showing green and black colorations were also more affected by Biotin R treatment. Our SEM-BSE observations highlight the need for a complete evaluation of treatment effects including those produced on both epilithic lichen thalli and their penetrating components and on epilithic and endolithic microbiota. The causes and consequences of the different efficacy of treatments observed and the potential risks of recolonization by viable cells left behind after treatment are here discussed.

1. Introduction

Damage to buildings and monuments caused by biological growths (mosses, lichens, fungi, alga, cyanobacteria, and non-photosynthetic bacteria), particularly that affecting recently cleaned buildings, is a cause of serious concern (Warscheid and Braams, 2000; De los Ríos and Ascaso, 2005; Young et al., 2008). Microbial attack not only causes aesthetic changes to the stone surface (epilithic lichens and/or or mosses cover, green and/or dark coloration), but also produces mechanical and chemical damage affecting both the stone surface and inner zones. It is this damage to the inner structure of a building that could lead to its decay in the long term (Ascaso and Wierzechos, 1995; Ascaso et al. 1998a, 1998b; Lisci et al., 2003; De los Ríos et al., 2004, 2009; Young et al., 2008; Favero-Longo et al., 2009). Thus, to select an effective cleaning strategy, prior

knowledge is needed of the epilithic and endolithic microbiota colonizing the stone and any chemical and mechanical alterations induced by their presence and activity (Ascaso et al., 2002; Sterflinger and Sert, 2006; Salvadori and Urzì, 2008; De los Ríos et al., 2009; Polo et al., 2010). In addition, the influence of the type of stone in terms of its bioreceptivity to this colonization needs to be first established (Miller et al., 2006, 2010; Cámara et al. 2008; Nugari and Salvadori, 2008).

A diagnosis of severe biodeterioration could indicate the need for treatments aimed at preventing favourable growth conditions for the most damaging microbiota or at directly inhibiting or eliminating them. In effect, microbial abatement is commonly achieved using biocides. Since biocides are toxic compounds, it is very important to identify the causative biodeteriogens and to choose a suitable product that targets only specific taxa (Polo et al., 2010). Biodeterioration treatments include both mechanical and chemical methods. An efficient treatment should kill damaging organisms and prevent their re-growth for an acceptable length of time while causing no damage to the substrate or the deposition of chemical residues in the stone (Young et al, 2008). Possible treatments should be tested on a small scale, but preferentially on the affected monument itself to determine their effectiveness against microorganisms colonizing different microhabitats. The effectiveness of biocides on microorganisms associated with the lithic substrate can be reduced significantly, compared to the sensitivity of microorganisms observed in lab efficacy tests (Warsheid and Braams, 2000; Nugari and Salvadori, 2008). Identifying innovative tools for testing the effects of biocides on both surface biodeteriogens and those occupying endolithic microhabitats is an important goal in the area of stone conservation. The most frequently used methods in this type of test have been those based on detecting changes in the chromatic and/or physical-chemical properties of the stone surface or biomass or identifying the reduced activity of the microorganisms colonizing

these areas (Monte and Nichi, 1997; Gorbushina et al., 2003; Tomaselli et al., 2003; Prieto et al., 2004; Cappitelli et al., 2007; Tretiach et al., 2007; Sanmartin et al., 2010). These methods, although they provide interesting information regarding effects on epilithic microbiota, are not useful for identifying treatment effects on endolithic microorganisms, most of which are also active deteriorating agents. Biomolecular approaches have been successfully used to determine changes in endolithic community structure in response to biocide treatments (Polo et al., 2010; Cámara et al., 2011) but these changes cannot be associated with a specific microhabitat. In contrast, the non-invasive method of detecting chlorophyll *a* fluorescence provides useful information on the effects of biocides on endolithic lichen photobionts without separating them from their microhabitat (Tretiach et al., 2010). Further, the backscattered electron scanning microscopy technique, SEM-BSE, allows assessment of treatment effects on photosynthetic or non-photosynthetic microorganisms both on and within the lithic substrate (Ascaso et al., 2002; Cámara et al., 2011). This procedure also serves to detect relict endolithic microhabitats where life forms in the stone persist after treatment. Thus, SEM-BSE is perhaps the best method of determining the effectiveness of a given treatment, since organic and mineral phases can be jointly examined without disturbing microhabitats and all the microbiota present can be ultrastructurally viewed (Ascaso et al., 2002; De los Ríos and Ascaso, 2005).

The late-Gothic cloister of the Segovia Cathedral (1472-1483) is the work of the architect Juan Guas. The cloister formed part of the old cathedral, which was replaced with the current Segovia Cathedral in 1530. For its construction, dolostones from the Segovian quarries of Bernuy and Casla were employed and in later additions, Sepúlveda stone and granite were used (Merino de Cáceres, 1996). In the old cloister, various signs of biodeterioration are appreciable. Some areas are completely covered by lichens and/or

mosses and detached stone fragments are frequent observed in colonized areas. The aim of the present study was to characterize the effects of two different cleaning procedures (biocide treatment followed by mechanical cleaning) against the biodeteriogens associated with the biodeterioration signs observed. Our ultimate goal was to extend our knowledge of the resistance to biocides certain microorganisms show owing to their specific microhabitat, and to design strategies to similarly preserve affected stone monuments.

2. Material and methods

2.1. Materials

The cloister showed some areas that are extensively colonized by lichens or mosses and others with black and green coloration. The most evident biodeterioration signs were observed at the bases of pillars (Fig. 1A-B), and along mouldings (Fig. 1C) and the lower parts of buttresses (Fig. 1D). These colonized areas were classified according to the type of biological colonization observed as:

I, areas colonized extensively by crustose lichen thalli (Fig. 1A) at the bases of pillars. The following species were the most abundant in these cloister areas: *Verrucaria nigrescens* Pers., *Lecanora dispersa* (Pers.) Rohl., *Caloplaca decipiens* (Arnold) Blomb. & Forsell, *Diploicia canescens* (Dicks.) A. Massal., *Lecidella stigmatea* (Ach.) Hertel & Leukert, and *Aspicilia contorta* (Hoffm.) Körb.

II, areas showing poorly developed crustose lichen thalli and green and black colorations due to unicellular cyanobacterial colonization (Fig. 1B) in the lower parts of buttresses. The most common lichen species detected were *L. dispersa*, *C. decipiens*, *D. canescens* and *V. nigrescens*.

III, areas showing the foliose lichen *Phaeophyscia orbicularis* (Neck.) Moberg and poorly developed mosses (Fig. 1C) mainly on mouldings.

IV, areas extensively colonized by mosses (Fig. 1D) mainly in lower parts of buttresses.

Lichen species were identified according to current methods and floras, as described by Clauzade and Roux (1985). The nomenclature used for the fungi follows the Mycobank database (<http://www.mycobank.org/>). The morphological terminology used for lichenized fungi follows that described by Barreno and Pérez-Ortega (2003).

Two types of carbonate rocks were identified in the material examined. Areas I and II were comprised of a fine-grained dolostone and areas III and IV of a bioclastic dolostone.

2.2. Cleaning procedures

Two different cleaning procedures involving the use of two different biocides followed by mechanical cleaning were used to remove the biodeteriogens detected in the different colonized areas described above. The selected chemicals were Biotin R and Biotin T (C.T.S. Spain). Both are broad-range biocides. Biotin R has a low solubility in water and comprises a mixture of iodopropynyl butylcarbamate and n-octyl-isothiazolone dissolved

in 2(2-butoxy-ethoxy ethanol). This compound was applied as a 5% solution in White spirit as per the manufacturer's instructions with a paintbrush until the entire surface was thoroughly saturated with the biocide solution. Biotin T, a water-soluble compound based on n-octyl-isothiazolone and a quaternary ammonium salt, was applied as a 2.5% solution in water as per the manufacturer's instructions also with a paintbrush until the entire surface was thoroughly saturated with the biocide solution. Both treatments were assayed in areas representative of the four types of biological colonization described above. In all the selected areas, one part was left untreated as a control. One week after biocide application, the treated surface was cleaned with a soft-bristled plastic brush soaked with the corresponding biocide solution to remove any remnants. Two months after treatment, representative samples of the different treated areas (3 from each sampling point) and their corresponding control areas were carefully obtained using a sterile scalpel without damaging the stone. Specifically, samples colonized by *V. nigrescens*, *A. contorta* and *Le. stigmataea* were obtained from area I, samples showing cyanobacterial colonization from area II, samples with *P. orbicularis* from area III and samples covered extensively by mosses from area IV.

2.3. Fluorescence microscopy

Fluorescence microscopy (FM) was used to assess the vitality of lichen symbionts after the different treatments. Tests were carried out on *V. nigrescens*, *L. dispersa*, *Le. stigmataea*, *A. contorta* and *P. orbicularis* and moss-colonized stone samples. The preparations were observed using a Zeiss AxioImager D1 fluorescence microscope and EC Plan-Neofluar 20x/0.50 and Plan-Apochromat 63x/1.40 oil-immersion objectives. A

CCD Axiocam HRc Rev 2 Zeiss camera and Carl Zeiss AxioVision 4.7 software were used to capture and record the fluorescence signals.

Lichen thallus and moss fragments were gently separated from the rock surface using a sterile blade. After brief fixation (2 h at 4 °C) in 2.5% glutaraldehyde they were stained with the SYTOX Green dead cell stain (S-7020, Molecular Probes). After 10 min staining at room temperature, small pieces of lichen thallus deposited on slides were examined by fluorescent microscopy using sets of filters: for DAPI (Zeiss Filter Set 49; Ex/Em: 365/420-470 nm), eGFP (Zeiss Filter Set 38; Ex/Em: 450-490/500-550 nm) and rhodamine (Zeiss Filter Set 20; Ex/Em: 540-552/567-647 nm).

2.4. Scanning electron microscopy with backscattered electron imaging

Lichen, moss and cyanobacteria colonized stone samples were prepared according to a procedure developed for observing the rock-microorganism interface by SEM-BSE (Wierzbos and Ascaso, 1994). The pieces of rock were fixed in glutaraldehyde and osmium tetroxide solutions, dehydrated in a graded ethanol series and embedded in LR-White resin. Blocks of resin-embedded rock samples were finely polished, carbon coated and observed using a DMS 960 SEM microscope. Microprobe analyses were performed using an energy dispersive spectroscopy (EDS) Link ISIS microanalytical system during SEM observation.

3. Results

On first inspection, the areas treated showed evident signs of cleaning of the stone surface after both cleaning procedures (biocide treatment followed by mechanical cleaning) compared to untreated areas (Fig. 1D-J, Fig. 2A-C). However, differences in the extent of cleaning were detected between the procedures, especially in areas with extensive crustose lichen cover (Fig. 1F-J). Biotin T procedure (Fig. 1J) generated a cleaner surface than Biotin R procedure (Fig. 1H). These differences were observed also using a magnifying glass, the Biotin R treated samples showing numerous remnants of epilithic lichens which were scarce in the samples treated with Biotin T. Fluorescence microscopy revealed that these remains of epilithic lichen colonization found after Biotin R treatment were groups of damaged cells, among which only a few algal cells maintained their autofluorescence (Fig. 2D-E). Differences in the extent of cleaning were also observed among species. Thus, in areas treated with Biotin R, remains of *V. nigrescens* and *A. contorta* were visible, but *Le. stigmatea* was almost abolished (Fig. 1G-H). In areas treated with Biotin T, lichen remains were lacking after the treatment and only a dark coloration corresponding to previous *V. nigrescens* colonization was detected (Fig. I-J). Differences in the extent of cleaning between the two procedures were not visibly evident in areas that had been colonized by cyanobacteria (Fig. 2A), mosses (Fig. 1D) or mosses and foliose lichens (Fig. 2B-C).

Using SEM-BSE, we were able to observe that crustose lichens in untreated areas were closely associated with the lithic substrate (Fig. 2F-G). In areas covered by these lichen thalli, mycobiont hyphae often penetrated inner dolostone zones (arrows in Fig. 2F) colonizing many fissures (arrows in Fig. 2G). In both cases, hyphae entered the rock

mainly through its intercrystalline porosity. Confirming our macroscopic observations, remains of epilithic lichen thalli were practically absent from the stone surface in samples treated with Biotin T (Fig. 3A-C). However, remnants of lichens with damaged cells were observed in samples treated with Biotin R (Fig. 3D). Fissures in zones of the lithic substrate previously colonized by *V. nigrescens*, *A. contorta* and *Le. stigmatea* were extensively occupied by living fungal hyphae with high amounts of lipids after treatment with Biotin T (Fig. 3A-C). However, living fungal hyphae were lacking (Fig. 3D-E) or scarce and only appeared in deep fissures in areas treated with Biotin R (white arrows in Fig. 3F). The remains of prior mycobiont colonization were observed in the fissures examined (Fig. 3E, black arrows in Fig. 3F). No differences in effects on mycobiont hyphae belonging to different crustose lichen species were detected.

Areas showing poorly developed crustose lichen thalli and greenish and blackish coloration (area II) were epilithically mainly colonized by aggregates of unicellular cyanobacteria (Fig. 4A). The presence of cyanobacteria aggregates was also detected in endolithic positions (arrows in Fig. 4A). Epilithic cyanobacteria were removed by both treatments (Fig. 4B-C). However, while endolithic undamaged cyanobacteria persisted after Biotin T treatment (arrows in Fig. 4B), samples from areas treated with Biotin R showed a high proportion of endolithic cells suffering plasmolysis and a loss of cell integrity (arrows in Fig. 4C).

Areas colonized preferentially by mosses and foliose lichens (area III) were observed on pillars and mouldings comprised of bioclastic dolostone. This stone also harboured numerous microorganisms, especially in intra-fossil pores but also between fossils. Under the foliose lichen *Phaeophyscia orbicularis* and mosses, fossil cavities left by the fossils were occupied by fungal hyphae, green algae and cyanobacteria mixed with mineral fragments (arrows in Fig. 4D). Biotin T procedure did not affect these microorganisms

occupying superficial cavities (Fig. 4E) and Biotin R procedure only showed a partial effect (Fig. 4F). Under dead fungal masses, we observed living fungal hyphae with high amounts of lipids (arrows in Fig. 4F and the corresponding enlargement) after treatment with Biotin R.

Lichen thalli and their penetrating hyphae could be related to different alteration processes in the stone in untreated areas. The surface lichen thallus affected the surface of both dolostone types (Fig. 2F-G, Fig. 4D). Thus, the dolostone colonized by the thalli of crustose lichens (*V. nigrescens*, *A. contorta* and *Le. stigmatea*) showed clear signs of surface damage. Crustose thalli fragmented the stone surface, separated mineral fragments and trapped them in the thallus (Fig. 2F-G). The hyphae of the foliose lichen *P. orbicularis* colonizing bioclastic dolostone could also be seen to penetrate the lithic substrate, causing disaggregation of the stone surface (Fig. 4D). EDS analysis confirmed the substrate origin of the mineral fragments present in the *P. orbicularis* thalli (Fig. 5A). In addition, the penetrating fungal hyphae were observed to grow through the intercrystalline porosity of the dolostone under the thallus, causing disaggregation of the stone surface and increasing its porosity (Fig. 2G). Biomobilization processes were not detected in association with endolithic dolostone growth (Fig. 5B), indicating mainly the mechanical actions of these hyphae. In bioclastic dolostone, microorganisms growing between fossils, especially fungi, seemed also to promote disaggregation phenomena (Fig. 4D).

4. Discussion

260 This study reveals the different efficacy of the two cleaning procedures tested against
261 superficial epilithic lichen thalli and their penetrating parts and the higher resistance of
262 the cells localized within the stone to both these treatments. It should be noted that the
263 cleaning procedure involving Biotin T treatment, which on visual inspection was found to
264 achieve better surface cleaning, nevertheless gave rise to living fungal hyphae in fissures
265 of the stone two months later indicating that the treatments were ineffective against these.
266 This emphasizes the need for a proper diagnosis using techniques capable of determining
267 the effects of treatment on lichen thalli and their penetrating components followed by an
268 assessment of the persistence of epilithic and endolithic forms or their recolonization
269 after treatment.

270 The effectiveness of biocide treatments against biodeteriogens depends on the methods
271 and products used on damage-causing species and their stage of development (Nugari and
272 Salvadori, 2003, 2008). In view of our results, besides this, the efficacy of treatment is
273 also highly dependant on the position occupied by the biodeteriogens in the stone. In our
274 study, both cleaning procedures proved effective at eliminating superficial epilithic lichen
275 thalli and living forms were not detected after treatment. However, Biotin T procedure
276 produced a more visually clean surface in areas colonized previously by crustose lichens
277 because of a lower presence of dead lichen thalli remnants on the surface of treated areas.
278 Mechanical cleaning with a solution of Biotin T in water therefore appeared to be more
279 effective at removing dead crustose lichen remnants than the solution of Biotin R in
280 White spirit. This differential effect is probably more related to a greater effectiveness of
281 water at removing the dead lichen thalli remnants during mechanical cleaning than to the
282 use of different biocides.

283 Through SEM-BSE we were able to evaluate treatment-induced changes not only to
284 epilithic lichens but also to their associated penetrating fungal hyphae. Both treatments

were shown effective in eliminating lichen thalli. This assessment also clearly indicated a greater effect on hyphae penetrating the lithic substrate of Biotin R procedure than Biotin T procedure. This different effectiveness of cleaning procedures could be related to the different accessibility of the biocides to the target organisms and to their persistence in the stone allowing them to act against the biodeteriogens for longer. The two biocides have different solubility properties such that different solvents are needed for their application. The solution in White Spirit seems to facilitate the action of the biocide on cells located in deep positions. The properties of this compound could promote the penetration and retention of the biocide solution in the stone and/or better absorption of the biocide by the microbial external cell wall. In addition, Biotin R is likely to remain for a longer period in the stone due to its low solubility in water, whereas the water-soluble Biotin T could more easily leach out of the stone. This factor could be especially important when treatments are targeted against endolithic cyanobacteria, which occupy small microhabitats close to the surface, where leaching of biocides could be facilitated. In effect, endolithic cyanobacteria showed a lower sensitivity to the water soluble Biotin T treatment. Both biocides are based on N-octyl isothiazolamine (OIT), which inhibits microbial growth and metabolism and induces irreversible cell damage resulting in loss of cell vitality (Williams, 2007; Gladis et al., 2010). Biotin R also contains iodopropynyl butylcarbamate (IPBC), which is insoluble in water and has high bactericidal and antifungal activity. We cannot exclude that this compound could be at least partially responsible for the damage observed in the hyphae and cyanobacterial cells occupying endolithic positions after Biotin R treatment.

The microbiota of bioclastic dolostones covered by foliose lichens and mosses was less affected by the cleaning procedures. This could be explained by the different type of porosity of the bioclastic dolostone (mainly interparticle and intra-fossil porosity) and the

probably lower accessibility of the biocide to the endolithic microhabitats. In addition, the special features of this kind of substrate with multiple internal and superficial cavities makes mechanical elimination more difficult after biocide actions on the remains of colonization occupying superficial cavities. Hence, these remains can act as a barrier to endolithic biocide penetration, reducing their efficacy. A similar barrier effect of lichens was described by Cámara et al. (2011) who examined the effectiveness of biocide treatments in stone from a dolostone quarry.

The deteriogenic activity of surface epilithic lichen thalli could be erroneously considered more dangerous, since visible aesthetic changes appear on the stone surface. However, our findings highlight the risks of assessing cleaning treatments only in terms of their surface cleaning effects without examining the presence and sensitivity of penetrating hyphae. Thus, endolithic microorganisms, mainly lichenized and free living fungi, are also important deteriorating agents as can be observed in the images presented here and as pointed out by several authors (Sanders et al., 1994; Ascaso et al., 1998a; De los Ríos et al., 2004, 2009; Cámara et al., 2008; Favero-Longo et al., 2009; Miller et al., 2010). Fungal hyphae induce damage as they penetrate the stone, causing an increase in pore size and the separation of mineral fragments. This action could continue after treatment if the fungi are not fully eliminated or if viable fungi recolonize the fissures. In addition, during rock contraction and expansion due to hydration-dehydration cycles, such deterioration processes are also favoured. The death of lichens as a consequence of biocide treatment could also generate tensions likely to accelerate the detachment of mineral fragments in colonized areas.

The effects of biocide treatment are always transient since treatment can only reduce the biota to a minimum but cannot prevent the revitalization of microorganisms not reached by the biocide (Sterfinger and Sert, 2006). The persistence of viable forms in

stone fissures resisting treatment could promote lichen and/or fungal recolonization of the rock making surface cleaning only effective over a short period. In addition, in the best scenario when all life forms are effectively eliminated, the presence of damaged and dead cells, especially in endolithic zones not susceptible to washing away by rainfall and the build up of this organic matter could be a source of nutrients for re-colonization or the growth of new lithobiontic microbiota, mainly saprotrophic fungi and non photosynthetic bacteria (De Leo and Urzi, 2003; Tratebas, 2004). The biocide leaching process progressively reduces the biocide content of the stone, allowing microorganisms from the environment to colonize surfaces. The rate of recolonization will not only depend on environmental factors such as temperature and humidity (Nugari and Salvadori, 2008; Shirakawa et al., 2010), but also on the bioreceptivity of the stone (De Leo and Urzi, 2003; Young et al., 1995). Repeat treatments could serve to completely avoid recolonization but this is not recommended for artwork since most methods have a mechanical component which can have detrimental effects on the substrate and can also cause chemical contamination (Nugari and Salvadori, 2008). If we are to adequately conserve our Cultural Heritage, new strategies need to be developed to eliminate microbial deteriogens with minimum risks for the monument and its environment while avoiding lichen and microbial re-colonization after treatment.

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488 Figure legends

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490 **Fig. 1:** A, pillar base colonized by different crustose lichen thalli from area I. B, pillar
491 base colonized by poorly developed lichen thalli showing green and black coloration due
492 to cyanobacterial colonization from area II. C, moulding colonized by *Phaeophyscia*
493 *orbicularis* thalli and poorly developed mosses from area III. D, lower part of a buttress
494 colonized by mosses after treatment (area IV). E, F pillar base colonized by crustose
495 lichens (area I) after the two cleaning procedures. Co (control, untreated), BR (Biotin R
496 treatment), BT (Biotin T treatment). Arrows indicate the areas observed in G-J. Detail of
497 pillar base indicated by the black arrow in Fig. 1F before (G) and after Biotin R
498 procedure (H). Detail of pillar base indicated by a grey arrow in Fig. 1F before (I) and
499 after Biotin T procedure (J). V (*Verrucaria nigrescens*), L (*Lecidea stigmatea*), A
500 (*Aspicilia contorta*).

501

502 **Fig. 2.** A, pillar base from area II after the two cleaning procedures. BR (Biotin R
503 treatment), BT (Biotin T treatment). B, Moulding from area III after Biotin R procedure.
504 C, Moulding from area III after Biotin T procedure. D, E Fluorescence microscopy image
505 of lichen symbiont cells in an *A. contorta* thallus from the control area (D) and *A.*
506 *contorta* remnants persisting after Biotin R treatment (E). F-G: SEM-BSE images of the
507 epilithic lichen thalli-lithic substrate interface in untreated areas. F, *V. nigrescens*. G, *A.*
508 *contorta*. Arrows indicate fungal penetration.

509

Fig. 3. SEM-BSE images of dolostone samples from areas colonized by crustose lichens (area I) after cleaning procedure with Biotin T (A-C) and Biotin R (D-F). A, zone colonized by *V. nigrescens* thalli after treatment showing fissures colonized by fungal hyphae. B, zone colonized by *Le. stigmathea* thalli after treatment showing intercrystalline colonization by living fungal hyphae. C, zone colonized by *A. contorta* after treatment showing a lack of epilithic lichen remnants and presence of living fungal hyphae in the stone's fissures. D, zone colonized by *V. nigrescens* after treatment showing remnants of dead lichen thalli and a lack of living forms in the stone's porosity (arrows). E, zone colonized by *A. contorta* after treatment showing remnants of dead fungal cells in pores. F, zone colonized by *Le. stigmathea* thalli after treatment showing deep fissures harbouring a mixture of dead (black arrows) and living fungal hyphae (white arrows).

Fig. 4. A-C SEM-BSE images of samples from areas showing green and dark coloration (area II). A, Extensive epilithic and endolithic (arrows) cyanobacterial colonization in untreated samples. B; presence of living cyanobacteria (arrows) in endolithic positions after Biotin T treatment. C, lack of epilithic cyanobacteria and internal fissures mainly occupied by damaged cyanobacteria cells (arrows) after Biotin R treatment. D, lichen-lithic substrate interface of the foliose lichen *Phaeophyscia orbicularis* colonizing bioclastic dolostone showing underlying endolithic colonization of fossil cavities (arrows). E, endolithic colonization in samples colonized by mosses two months after Biotin T treatment. F, living fungal forms (arrows) in fissures under dead lichen thalli in areas colonized by mosses and treated with Biotin R. In the top corner, an enlarged image is provided of the zone indicated with the white arrow, showing living fungal cells with high amounts of lipids.

534

535 **Fig. 5.** A, *Phaeophyscia orbicularis* colonizing bioclastic dolostone. EDS spatial
536 distribution of Mg in the zones indicated by the box in Fig. 4D, revealing the lithic substrate
537 origin of mineral fragments. B, EDS spatial distribution of Mg and Ca in a dolostone area
538 colonized by penetrating mycobiont hyphae after Biotin T treatment.

Fig. 1

Fig. 2

Fig. 3

20 μm

Fig. 5