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Running title

LASER DAMAGE TO *Verrucaria nigrescens*

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Abstract

Epilithic and endolithic microorganisms and lichens play an important role in stone biodeterioration. The structural and physiological damage caused by nanosecond pulsed laser of 1064 nm from Nd:YAG laser to *Verrucaria nigrescens* lichen as well as to endolithic algae and fungi were investigated in the present study. Ultrastructural laser effects on lichen and endolithic microorganisms were study without disturbing the relationship between lichen and lithic substrate by taking lichen-containing rock fragments and processing both together. SEM-BSE, LT-SEM and FM were used to determine cell integrity and ultrastructure, which reflect microorganism viability. Photobiont vitality was determined using a PAM chlorophyll fluorescence technique. The lichen thalli were completely removed by irradiation with 5 ns pulses at a fluence of 2.0 J/cm^2 with no stone damage as showed by Micro-Raman spectroscopy. The fungal and algal endolithic cells located below were completely destroyed or presented a high plasmolysis degree resulting from heating their microenvironment. The lichen and endolithic mycobiont near the irradiated zone were also damaged. Algal photosynthetic damage prevents fungal survival and lichen viability. This is the first report of laser removal and inactivation of lichen and lithic microorganisms, and thus provide an environmentally-friendly and efficient method to control stone biodeterioration.

Keywords: biodeterioration, stone, laser, algae, fungi, lichen

1. Introduction

Diverse groups of microorganisms such as heterotrophic bacteria, cyanobacteria, free-living algae and fungi are responsible for biodeterioration in stones used to construct heritage buildings and monuments (Caneva *et al.*, 2008). Lichens, a symbiotic association of a fungus (the mycobiont) and a photosynthetic partner, usually an alga and/or cyanobacteria (the photobiont), are also common colonizers of monumental stone. These biological agents can colonize the surface of the lithic substrate (epilithic colonizers) as well as the internal zone of the stone (endolithic colonizers), where they develop complex interactions with the mineral substrate (Ascaso *et al.*, 1998a; Ascaso *et al.*, 1998b; Ascaso *et al.*, 2002; Caneva *et al.*, 2008). Both types of colonizers play an important role in stone biodeterioration, being responsible for several chemical and physical processes that enhance weathering reactions (Ascaso *et al.*, 1998a; De los Ríos and Ascaso, 2005). The presence of endolithic microbial forms and their relationship with weathering effects on stone was proven by using different *in situ* microscopy techniques (Ascaso *et al.*, 1998a; Ascaso *et al.*, 2002).

Current control of lichens and microorganisms involved in stone biodeterioration processes includes chemical and physical treatments (Caneva et al., 2008; Doehne and Price, 2010; Scheerer et al., 2009). However, in spite of the great diversity of protocols currently in use in the field of stone conservation, methods to control biodeterioration have proven their technical and environmental limitations (Alakomi et al., 2006; Bartolini et al., 1999; Caneva et al., 2008; Doehne and Price, 2010; Scheerer et al., 2009). In this sense, further research is necessary on the effects on the substrate and the efficiency of physic methods to control stone-deteriorating microorganisms (Scheerer *et al.*, 2009). Furthermore, chemical methods have limitations as biocide' low substrate penetration, certain side effects such as irreversible stone damage and/or

low retention in stone that often limit their action (Caneva *et al.*, 2008; Doehne and Price, 2010). Therefore, short-term applications must be repeated (deCruz *et al.*, 2005). Scanning electron microscopy in back-scattered electron mode (SEM-BSE) technique showed that in some cases biocide action to control epilithic and endolithic microorganisms was limited when applied either alone or combined with mechanical pretreatments (Cámara *et al.*, 2011; De los Rios *et al.*, 2011). In recent decades chemical biocides were banned because of toxicity-related environmental and health hazards (European-Comission-Regulation, 2007; SCENIHR, 2009). Consequently, these more restrictive environmental regulations have led to a growing need for safe, efficient, and cost-effective treatments to remove and control microorganisms and lichen that avoid the disadvantages involved by traditional management of stone biodeterioration processes(European-Comission-Regulation, 2007).

Laser irradiation is used as a successful procedure to control human pathogenic microorganisms such as pathogenic bacteria, keratinophilic, and dermotophitic fungi (Burns et al., 1993; Manevitch et al., 2010; Smijs and Schuitmaker, 2003; Vural et al., 2007). Laser treatment could be used for antifouling purposes, causing mortality to biofilm-forming microorganisms (Nandakumar *et al.*, 2002; Nandakumar *et al.*, 2004). In the field of cultural heritage, laser cleaning is a well-established technique because it provides fine and selective removal of superficial deposits and encrustations such as biological and black crust(Cooper, 1998; Gomoiu et al., 2006; López et al., 2010; Marakis et al., 2000; Maravelaki-Kalaitzaki et al., 2003; Oujja et al., 2005; Potgieter-Vermaak et al., 2005; Pouli et al., 2008; Tornari et al., 2006). Nowadays, portable laser cleaning systems can remove contaminants from substrates with no surface damage, thus being a great solution for many conservation projects. These systems allow a cost effective use of laser technology to conserve large objects such as buildings or large sculptures

(Pouli *et al.*, 2011). However, despite the widespread use of lasers in conservation, few laser cleaning studies have been focused on the removal of biodeterioration agents such as epilithic lichen and fungi from stone (deCruz et al., 2009; deCruz et al., 2005; Sarantopoulou et al., 2006; Speranza et al., 2011). Furthermore, most of these researches mainly rely on the chemical/physical aspects of the processes (deCruz et al., 2009; deCruz et al., 2005)or the irradiation of fungi previously isolated from deteriorated stone parts and cultured in artificial media that differed greatly from the stone substrate (Sarantopoulou *et al.*, 2006).

In this work, we study the degree of structural and physiological damage to mycobiont and photobiont cells from *Verrucaria nigrescens* thalli as well as fungi and algae inside the stone caused by nanosecond pulsed laser of 1064 nm from Nd:YAG laser. To the best of our knowledge, this is the first study on the effect of laser on lithic microorganisms growing on the surface and inside the stone.

2. Material and methods

2.1. Samples collection, chlorophyll a fluorescence (Chl_aF) measurements, and selection of vital lichen thalli

Lichen thalli on dolostone were collected from the Redueña quarry (917 m above sea level; N 40° 47.66′; O 3° 33.25′, Madrid, Spain). *Verrucaria nigrescens* thalli were identified according Clauzade and Roux (1985), and Nimis and Martellos (2008).

In order to select *V. nigrescens* thalli with active photobiont, Chl_aF measurements were taken in the field as previously described (Schroeter *et al.*, 1999; Tretiach *et al.*, 2010). The potential maximal photosystem II quantum yield (Fv/Fm= variable fluorescence/maximal fluorescence) is

an indicator of lichen vitality and was determined with MiniPAM (Walz, GmbH,FRG) in fully-hydrated and dark-adapted thalli (Tretiach *et al.*, 2007). Dolostone samples with healthy *V. nigrescens* thalli were taken to the laboratory and kept under controlled low-humidity conditions for 24 hours. Samples with *V. nigrescens* epilithic thalli and endolithic colonization were selected for laser treatment using a stereomicroscope. Before laser irradiation (a maximum of 72 h after sample collection), Chl_a F measurements were repeated to ensure lichen vitality.

2.2. Laser laboratory treatments

Laboratory laser irradiations were performed on the selected hydrated stone samples (fourteen zones), colonized both by *V. nigrescens* and endolithic colonization, using a Q-switched Nd:YAG (neodymium-doped yttrium aluminum garnet) laser (Quantel Brilliant B) that delivered pulses of 5 ns full width half maximum (FWHM). The unfocussed laser beam was directed to the surface of the sample with the help of mirrors. Irradiation was performed at the fundamental wavelength (1064 nm) with a fluence of 2.0 J/cm^2 at a 10 Hz repetition rate, delivering 100 pulses in an area of ca. 0.5 cm^2. Pulse energy was measured by a joulemeter (Gentec ED-200). The selected laser fluence was just below the stone ablation threshold and determined in previous experiments evaluating 19 laser irradiation conditions (Speranza *et al.*, 2011). Immediately after irradiation, the colonized-stone samples were prepared for observation under the stereomicroscope and electron microscopy.

2.3. Stereomicroscopic observations

Morphological changes produced in the lichenized microorganisms as well as in endolithic microorganisms after irradiation were observed with a Leica S8APO stereo-microscope equipped with a Leica EC3. The images were taken at different magnifications using LAS EZ software.

2.4. Fluorescence microscopy

Fluorescence microscopy (FM) was used to assess vitality of lichen symbionts after the different treatments. The preparations were observed using a Zeiss AxioImager D1 fluorescence microscope (Carl Zeiss, Jena, Germany) Plan-Apochromat 63×/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 fluorescence signals. Fragments were taken from sound lichen thalli, and lichen thalli next to the irradiated cleaned surface were gently separated from the rock surface using a sterile blade. After short fixation (2 h at 4 ºC) in 2.5% glutaraldehyde, they were stained with the SYTOX Green dead cell stain (S-7020, Molecular Probes). The SYTOX Green dye is a simple and quantitative dead-cell indicator, a high-affinity nucleic acid stain that penetrates only cells with damaged plasma membranes (i.e., dead cells) (Wierzchos *et al.*, 2004). The original solution containing 5 mM SYTOX Green in anhydrous DMSO was water diluted 1:100 and added to fragmented lichen thallus. After 10 min counterstaining at room temperature, small pieces of lichen thallus deposited on slides were examined by fluorescent microscopy using sets of filters: for 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). Signals from both channels were recorded: a green signal (blue excitation; 38 filter set) that reveals nuclear DNA structures in damaged algal and fungal cells, and a red emission signal (green excitation; 20 filter set) that reveals red auto-fluorescence emitted by the chlorophyll of algal phycobiont cells**.** To obtain 3D images, 15 sections (0.5 μm between them) were recorded by Z-stack capture and multichannel image acquisition. The obtained images were 3D reconstructed using software package AXIOVISION 4.7 and represented as maximum intensity projection (MIP) images.

157 *2.5. Scanning electron microscopy*

158 To determine the laser effect at cytological level, the structure of the epilithic lichenized
159 microorganisms and the microorganisms found in the endolithic zone were examined before and
160 after laser irradiation by different scanning electron microscopy techniques. Control and
161 irradiated stone (five and seven samples respectively), colonized by lichen and microorganisms
162 were processed for SEM-BSE observation according to a method introduced by Wierzchos and
163 Ascaso (1994). Laser irradiated samples included: the cleaned stone surface area (removed lichen
164 thalli) and the neighboring areas containing apparently healthy *V. nigrescens* thalli. Briefly, the
165 stone samples covered by *V. nigrescens* and containing endolithic microorganisms were cut
166 transversely and fixed with 3.25% glutaraldehyde in 0.05 M cacodilate buffer, and subsequently
167 stained using a 1% OsO_4 solution in 0.025 M cacodilate buffer. After fixing, the samples were
168 dehydrated in a series of ethanol solutions, embedded in LR^{TM}White resin, and fine-polished after
169 polymerization. The fine-polished surfaces of the stone sample cross-sections were carbon coated
170 and finally examined using a DMS 960 Zeiss SEM equipped with a four-diode, semiconductor
171 BSE detector at ICA-CSIC facilities (Madrid, Spain). Control and irradiated stone surfaces
172 colonized by *V. nigrescens* (four samples of each type) were air dried, carbon coated, and
173 observed using a DMS 960 Zeiss SEM in secondary electron mode (SE).

174 *2.5.3. Low temperature scanning electron microscopy (LTSEM)*

175 Lichen thalli (three irradiated and two non-irradiated) were examined using the LTSEM
176 technique (De los Ríos and Ascaso, 2005). Small sample fragments were mounted using OCT
177 (Optimal Cutting Temperature) compound (Gurr), and mechanically fixed onto the specimen
178 holder of the cryotransfer system (Oxford CT1500). Subsequently, the samples were plunge-

frozen into subcooled liquid nitrogen, and then transferred to the preparation unit. There, the frozen specimens were cryofractured and etched for 2 min at -90°C. After ice sublimation, sample surfaces were gold sputter coated and then transferred onto the cold stage of the SEM chamber. Fractured surfaces were observed under a DSM960 Zeiss SEM microscope at -135°C.

2.6. Micro-Raman spectroscopy analyses

Micro-Raman spectroscopy was used to detect possible structural and chemical changes on irradiated areas. Raman analyses were carried out using a Thermo Fischer DXR Raman microscope (West Palma Beach, FL 33407, USA), whose point and shoot spatial resolution capability was 1μm. The spectra were taken with 4 cm^{-1} resolution and 20-s exposure using the 10x objective of the confocal microscope (spot size 2 μm). A laser emitting at 532 nm with 10 mW power was used as an excitation source. OMNIC 1.0 software package was used for system operation.

3. Results

All photobiont from lichen thalli used for laser irradiation were photosynthetically active in the field and under the laboratory conditions, Fv/Fm values ranging between 0.60-0.79. The small dark brown crustose thallus of *V. nigrescens* showed the typical aspect of this epilithic lichen (Fig. 1A-B). *V. nigrescens* produces no substance responsible for stone darkening, but the natural color of the dolomite stone is masked by the thalli and, as a consequence, the substrate looks rather dark (FIG. 1A-B).

Stereomicroscope examinations proved that treatment conditions were very effective in removing *V. nigrescens* thalli (Fig. 1A). The laser irradiated area had been cleaned, and the fungi that

200 composed the lichen basal layer had been efficiently removed (Fig. 1A, C and D). Although
201 green colour from algae is clearly visible on the surface by stereo microscopy (Fig. 1C),
202 remnants composed by severely damaged algal and fungal cells could be observed on the
203 dolostone surface by SEM-SE (Fig. 1 D-E).

204 These remnants are mainly formed by broken and empty fungal hyphae that showed altered cell
205 walls (Fig. 1E). Several laser cleaned areas, as shown in Figures 1A and D, were analyzed using
206 micro-Raman spectroscopy. The micro-Raman spectra of the dolomite stone acquired in control
207 and stone areas colonized by *V. nigrescens* thalli before and after irradiation are shown in Figure
208 2, and Table 1 lists the bands observed, together with their corresponding assignments. The
209 spectrum of the *V. nigrescens* control thalli (non irradiated) presents the characteristic bands of
210 disordered and graphitic carbon, centred at 1340 and 1580 cm^{-1} respectively, which are related to
211 the presence of organic compounds. When *V. nigrescens* thalli are irradiated, the spectra of the
212 laser cleaned areas display the characteristic bands of calcium carbonate, as it can be observed in
213 the dolomite control sample, which correspond to the vibration modes of the free CO_3^{2-} ion of
214 calcium carbonate. These bands are attributed to lattice vibrations at 175 and 299 cm^{-1}, and to in-
215 plane bending and symmetric stretch modes at 727 and 1097 cm^{-1}, respectively. The spectrum
216 also displays bands assigned to the hydrocarbons present in the dolomite stone at 1440 cm^{-1} (ν_s
217 (C-O) + ν (C-C) vibration mode), 1615 cm^{-1} (ν_a (C-O) vibration mode), and 1744 cm^{-1} (ν(C=O)
218 stretching).

219 In general, irradiated stone showed similar aspect to the control, and fissures, fractures or
220 porosity changes were not observed in the irradiated surface or internal zones of the stone (Fig. 3
221 A, C,E; Fig. 4 A-D and Fig. 5A-D) when observed by SEM-BSE and SEM-SE. The anatomy of

222 the epilithic lichen thalli and the cytology of photobiont and mycobiont, and the microorganisms
223 in endolithic zones before and after laser irradiation were examined by SEM-BSE and LTSEM
224 (Fig. 3-5). Thin to moderately thick *V. nigrescens* thalli of 50 to 250 μm, including the basal
225 layer, were observed on the superficial layer of the dolostone (Fig. 3A). A typical thallus presents
226 a poorly differentiated upper cortex of variable width composed of thick, densely packed fungal
227 cells (2.5-3.0 μm), a photobiont layer formed by green algae (6.0-9.0 μm in diameter), and
228 mycobiont cells (3.0-6.0 μm in diameter) (Fig. 3B). The mycobiont hyphae showed several small
229 lipid globules occupying a substantial portion of the protoplast volume (Fig. 3B). In Fig. 3A and
230 C the medullary region can be seen, with abundant hyphae of 7-12 μm in diameter that have
231 expanded through fissures reaching deep stone areas (up to 200 μm). The cells of fungal hyphae
232 were entirely occupied by lipids (oil cells). As lipids are very osmiophilic when inside a cell
233 previously treated with osmium, a strong signal due to the lipids' osmium uptake is detected
234 (Fig. 3C box). Other fungal clusters near the lichen thallus also showed high cytoplasmatic lipid
235 content (Fig. 3D).

236 Laser irradiation left empty spaces in the dolomite surface that correspond with the spaces
237 occupied by the lichen thalli before treatment. These can reach 80 μm from the surface (Fig. 3E
238 asterisks). The microorganisms found in endolithic zones were also altered by laser surface
239 irradiation as shown by SEM-BSE analyses (Fig. 3 E-F and Fig. 4). Most fungal cells near the
240 stone surface were completely destroyed (Fig. 3 E arrows) or showed high plasmolysis degrees
241 (Fig. 3E box and F).

242 Fungal cytoplasm looked sponge-like and the lipid bodies could only be distinguished in some
243 cells (Fig. 3 F). Free fungi on the surface and endolithic colonization forms were also observed in

split longitudinal sections of the dolostone using the SEM-BSE technique (Fig. 4A top box). In these samples, algal and fungal cell clusters were also frequently found randomly distributed in deep areas of the dolomite stone (Fig. 4A lower box). Fungi on the stone surface were removed by laser irradiation and the endolithic clusters were also severely affected (Fig. 4B). A similar but weaker effect was also observed in clusters of phototrophic and heterotrophic microorganisms in deeper stone areas, down to 1000 μm under the cleaned dolostone surface (Fig. 4C and D).

Laser irradiation also affected lichen thalli and microorganisms near cleaned areas (Fig. 5). The cell damage severity observed depends on the localization of lichen thalli from the laser irradiated zone. First, on the border of the cleaned areas, lichen thalli were observed to apparently keep their integrity (Fig. 5A). However, their algal and fungal cells had been strongly affected, as these cells were empty and only conserved their walls (Fig. 5A). Second, thalli with intermediate damage stages were detected near this area (Fig. 5B). In this case, in the lower part of the thalli some fungal cells with cytoplasm and lipid bodies remain altough with evident signs of damage (Fig. 5C). The endolithic mycobiont hyphae as well as fungi and algal cell clusters under the thalli (Fig. 5B box) were also affected by the laser irradiation applied in the neighbouring cleaned area. Laser-produced structural damage was evident when compared to control thalli observed by SEM-BSE (Fig. 5D) and further confirmed by LTSEM (Fig. 5E and F).

Fungi and algae from control *V. nigresecens* thalli with well-defined cell walls and cytoplasmatic content could be observed by LTSEM (Fig. 5E). Some ultrastructural cell details in lichen photobionts such as the chloroplast which often occupy most algal cell volume were also observed by LTSEM in the control sample (Fig. 5E box). However, lichen thalli close to the

265 cleaning zone that showed some apparent macroscopic integrity were actually formed only by
266 algal and fungal cell walls, as their cytoplasms had been totally destroyed (Fig. 5F).

267 In addition to the SEM-BSE and LTSEM results, fluorescence microscopy confirms that laser
268 irradiation also affected lichen thalli localized near cleaned areas. *V. nigresecens* thalli control
269 (Fig. 6A) and thalli in the border area and near irradiated areas (Fig. 6B) were stained with the
270 SYTOX Green Live/Dead dye. The red emission signal reveals autofluorescence signal emitted
271 by chlorophyll in algal phototrophic cells, while the green emission signal comes from the
272 SYTOX Green stained nucleic acids of photobiont and mycobiont cells with damaged plasma
273 membranes (arrows in Fig. 6B). While most algal cells in the control sample showed no green
274 signal (Fig. 6A), almost all algal cells from lichen thalli near the irradiated area reveal SYTOX
275 Green stained nucleic acids signal from cells with damaged plasma membranes (Fig. 6B).

276 **4. Discussion**

277 Nd:YAG laser treatment under the irradiations conditions used (1064 nm, 5 ns) was effective at
278 removing *V. nigrescens* hydrated active thalli producing no chemical changes in the irradiated
279 stone surface, as observed by micro-Raman, SEM-BSE, and SEM-SE analyses. *V. nigrescens* is a
280 crustose epilithic lichen that is reportedly a widespread species on limestone heritage buildings
281 and monuments throughout Europe (Blazquez *et al.*, 1995; Nimis and Martellos, 2008; Smith *et*
282 *al.*, 2010) and also colonizes extensive areas of the Redueña dolostone quarry face (Madrid,
283 Spain) (Cámara *et al.*, 2011). In addition, severe cellular damage was produced not only in the
284 endolithic mycobiont hyphae and neighbouring lichen thalli but also in fungi and algae deep in
285 the stone. Although identification of these microorganisms inside the stone was not carried out
286 because it was not the main aim of the present work, these microorganisms showed similar

287 morphologies to those previously detected by SEM-BSE and identified by molecular biology in a
288 nearby Redueña quarry face (Cámara *et al.*, 2011).

289 The Fv/Fm values measured in the photobiont microorganisms in the laboratory were consistent
290 with the range of values registered in other crustose lichens(Schroeter *et al.*, 1999; Tretiach *et al.*,
291 2010) These values fell within the range determined in the quarry face in the autumn season
292 when *V. nigrescens* is metabolically active, and confirmed the photobiont vitality of the selected
293 samples for laser irradiation experiments.

294 Lichens show different sensibility to biocides related to thallus structure and physiological state,
295 as well as to chemical biocide nature (Tretiach *et al.*, 2010). The main problem detected during
296 the biocide treatment of lichens encrusted in stone artworks was that considerable parts of the
297 thalli still remained attached to the stone after several weeks of application of highly concentrated
298 biocide with bristle brushes or poultices (Caneva et al., 2008; deCruz et al., 2005; Doehne and
299 Price, 2010). In previous experiments, after biocide application, a mixture of dead and living
300 microorganisms was found, these included living fungal forms in fissures and under dead lichen
301 thalli, and lichen thalli areas of no biocide action (Cámara *et al.*, 2011; De los Rios *et al.*, 2011).
302 In contrast, as shown in this and other works, laser cleaning produces complete lichen-thallus
303 removal, even if lichen debris on the surface can be brushed away without affecting dolostone
304 surface (deCruz et al., 2007; deCruz et al., 2009; Leavengood et al., 2000). In addition, if the
305 considerable cell damage observed in lichen debris, as well as in the endolithic mycobiont cells
306 and microorganisms in the deeper substrate areas is taken into account the possibility of stone
307 recolonization from theses sources is rather low. The collateral damage produced in lichen thalli
308 near cleaned areas is also important.

309 Laser application to lithic microorganisms and lichens without extracting them from their
310 substrate have been scarcely studied (deCruz et al., 2007; deCruz et al., 2009; Leavengood et al.,
311 2000). In general, the biological component of stone crust, known as "biological crust", is poorly
312 characterized, and commonly-used techniques enable no evaluation of laser-produced structural
313 damage and vitality changes. In this sense, SEM-BSE microscopy allows assessing laser effect
314 on lichen epilithic thalli and microorganisms in deep stone areas without extracting them from
315 their microhabitat (Ascaso et al., 1998a; Ascaso et al., 1998b; Ascaso et al., 2002; De los Ríos
316 and Ascaso, 2005; Wierzchos and Ascaso, 1994, 1996). SEM-BSE microscopy also provides
317 information on cell-wall integrity and some ultrastructural aspects of lithic microorganisms that
318 reflect their vitality *in situ* (Ascaso et al., 1998b; Ascaso et al., 2002; Cámara et al., 2011; De los
319 Ríos and Ascaso, 2005; De los Rios et al., 2004; De los Ríos et al., 2010; De los Rios et al., 2011;
320 Potgieter-Vermaak et al., 2005). In this way, the different degrees of laser-induced damage
321 undergone by epilithic and endolithic microorganisms were determined.

322 Laser effect on lithic microorganisms may depend on different factors, including cellular
323 structure, chemical composition, and location in the stone Lichen thallus anatomy, the presence
324 of a protective upper cortex, thick mycobiont cell walls, and intimate thallus-substrate
325 relationship may represent certain additional difficulties for the laser cleaning procedure. Under
326 our experimental conditions, laser ablation led to complete removal of *V. nigrescens* epilithic
327 hydrated thalli. However, the upper cortex of a different crustose lichen, *Diploicia canescens*,
328 was observed more resistant under the same laser irradiation conditions used for the removal of
329 *V. nigrescens* (Speranza *et al.*, 2011). Higher fluence is proven necessary for thallus removal in
330 this case. Even though Nd:YAG laser destruction of epilithic lichen and endolithic
331 microorganisms cannot be unequivocally ascribed to specific physical/chemical mechanisms,

certain ideas based on previous research can however be pointed out. Laser-removal efficiency depends on light-material interaction, which is in turn related to wavelength, fluence, and the optical properties of the target such as light absorption and heat diffusion (deCruz et al., 2009; Siano, 2007). The influence of these factors during laser irradiation of *Diploschistes scruposus* (a grayish-white crustose lichen) had been proven in a recent study (deCruz *et al.*, 2009). Moreover, the extent of laser cell damage depends on both fluence and time of exposure to laser light. In bacteria the laser effect could be enhanced by the presence of pigments (Manevitch et al., 2010; Maravelaki-Kalaitzaki et al., 2003; Vural et al., 2007). Therefore, a rapid heat transfer to lichen thalli could take place when *V. nigrescens* absorbs laser light, as described for other lichens and microorganisms (Cappitelli *et al.*, 2007; deCruz *et al.*, 2007). In this way, the local heating induced by laser irradiation at 1064 nm could also affect the thalli near cleaned areas. This phenomenon could be responsible for cytoplasm damage of photobiont and mycobiont cells, especially in the case of alteration of fungal lipid bodies. Although several authors report diverse morphology of lichen oil cells, their function still remains unknown (Gueidan et al., 2007; Kushnir, 1978; Pinna et al., 1998). Kushnir et al. (1978) state that oil hyphae do not occur in epilithic lichen. This fact is also supported by the interesting studies by Pinna et al. (1998).

These authors describe oil-hyphae using SEM in secondary mode (SEM-SE), which allows observing the outside of the oil cells, and point out that true-oil hyphae occur beneath epilithic thalli only when these overgrow endolithic species. In our study, SEM in backscattered mode (SEM-BSE) enabled us to observe lipids inside the cells, both in the control sample and the laser-treated sample. We can perfectly describe cells with lipids (oil cells) belonging to the epilithic thalli, but it is difficult to say if there are true-oil hyphae or fungal cells with lipid inclusion (Gueidan et al., 2007; Kushnir, 1978; Pinna et al., 1998). SEM-BSE observations allowed us to

355 observe alterations in the lipidic integrity inside the cells of the laser-treated samples. In
356 photobiont cells, damage to the photosynthetic system will also affect fungal survival and lichen
357 viability.

358 From a practical point of view, lichen mechanical removal is unadvisable in order to not promote
359 stone disintegrations in some circumstance. In this case, induced thermal damage of thalli could
360 be a useful strategy to stop lichen stone biodeterioration activity, especially if the severe
361 cytoplasmatic damage observed in the endolithic mycobiont hyphae is taken into account.

362 Moreover, certain resistance to the thermal laser effect was observed in mycobiont cell walls. The
363 walls are responsible for the mechanical strength and thermal resistance of fungal cells (Osherov
364 and Yarden, 2010). Furthermore, microenvironment heating could be responsible for the severe
365 damage observed in microorganisms in endolithic areas. Similar microenvironment heating
366 processes are responsible for the inactivation and death of unexposed microorganisms such as the
367 bacteria inside the dentine microcrystalline structure during Nd:YAG laser treatments (Goodis *et*
368 *al.*, 1993). A characteristic of such a laser system is that it reaches deeper stone areas of interest
369 when compared to UV rays and xenon lamps used against microorganisms and lichen on stone
370 that —in spite of their germicidal activity— also display poor penetration power (Doehne and
371 Price, 2010; Goodis et al., 1993; Leavengood et al., 2000).

372 **5. Conclusion**

373 All results shown indicate that irradiation with a Q-switched Nd:YAG laser is a promising and
374 environmentally friendly method to control lichen and microorganisms involved in stone
375 biodeterioration. Although, further studies are needed to elucidate the specific physical/chemical
376 mechanisms involved, the results obtained extend the traditional idea that laser irradiation can

only clean stone surfaces, thus proving an additional important laser effect related to the microbiota community involved in several deterioration mechanisms. The integration of SEM-BSE, LTSEM, FM, and micro-Raman analyses constitutes an effective strategy to determine the optimal laser irradiation regime to control both stone integrity and biological damage. Field experiments of laser irradiation of lichen thalli in the quarry are currently in progress and allow us to evaluate different parameters such as the biological recolonization of cleaned and irradiated surfaces.

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References

Alakomi, H.-L., Paananen, A., Suihko, M.-L., Helander, I.M., Saarela, M., 2006. Weakening effect of cell permeabilizers on gram-negative bacteria causing biodeterioration. Applied and Environmental Microbiology, 4695-4703.

396 Ascaso, C., Wierzchos, J., Castelló, R., 1998a. Study of the biogenic weathering of calcareous litharenite
397 stones caused by lichen and endolithic microorganisms. International Biodeterioration and Biodegradation
398 42, 29-38.

399 Ascaso, C., Wierzchos, J., Delgado Rodrigues, J., Aires-Barros, L., Henriques, F.M.A., Charola, A.E.,
400 1998b. Endolithic microorganisms in the biodeterioration of the tower of Belem. International Zeitschrift
401 für Bauinstandsetzen 4, 627-640.

402 Ascaso, C., Wierzchos, J., Souza-Egipsy, V., De los Rios, A., Delgado Rodrigues, j., 2002. In situ
403 evaluation of the biodeteriorating action of microorganisms and the efects of biocides on carbonate rock of
404 the Jeronimos Monastery (Lisbon). International Biodeterioration and Biodegradation 49, 1-12.

405 Bartolini, M., Pietrini, A.M., Ricci, S., 1999. Use of UV-C irradiation on artistic stone works for control
406 of algae and cyanobacteria. , In: Tiano, P.M., G. (Ed.), Microbes and Art: The role of microbial
407 communities in the degradation and protection of cultural heritage. CNR, Florence, pp. 221-227.

408 Blazquez, F., Calvet, F., Vendrellc, M., 1995. Lichen alteration and mineralization in calcareous
409 monuments of northeastern Spain. Geomicrobiology Journal, 223-247.

410 Burns, T., Wilson, M., Pearson, G., 1993. Sensitisation of cariogenic bacteria to killing by light from a
411 helium-neon laser. Journal of Medical Microbiology 38, 401-405.

412 Cámara, B., De los Ríos, A., Urizal, M., Álvarez de Buergo, M., Varas, M.J., Fort, R., Ascaso, C., 2011.
413 Characterizing the microbial colonization of a dolostone quarry: implications for stone biodeterioration
414 and response to biocide treatments. Microbial Ecology 62, 299-313.

415 Caneva, G., Nugari, M.P., Salvadori, O., 2008. Control of biodeterioration and bioremediation techiques.,
416 In: Caneva, G., Nugari, M.P., Salvadori, O. (Eds.), Plant biology for cultural heritage : biodeterioration
417 and conservation Getty Conservation Institute, Los Angeles pp. 309-346.

418 Cappitelli, F., Nosanchuk, J., Casadevall, A., Toniolo, L., Brusetti, L., Florio, S., Principi, P., Borin, S.,
419 Sorlini, C., 2007. Synthetic consolidants attacked by melanin-producing fungi: case study of the
420 biodeterioration of Milan (Italy) Cathedral marble treated with acrylics. Applied and Enviromental
421 Microbiology 73, 271-277.

422 Clauzade, G., Roux, C., Houmeau, J.M., Raimbault, P., 1985. Likenoj de Okcidenta Europo. Ilustrita
423 Determinlibro.

424 Cooper, M., 1998. Laser cleaning in conservation: an introduction, Oxford.

425 De los Ríos, A., Ascaso, C., 2005. Contributions of in situ microscopy to the current understanding of
426 stone biodeterioration. International Microbiology 8, 181-188.

427 De los Rios, A., Ascaso, C., Wierzchos, J., E., F.-V., Quesada, A., 2004. Microstructural characterization
428 of cyanobacterial mats from the McMurdo ice shelf, Antarctica. Applied and Environmental Microbiology
429 70, 569-580.

430 De los Ríos, A., Ascaso, C., Wierzchos, J., Sancho, L.G., 2010. Space flight efects on lichen ultrastructure
431 and physiology, In: Seckbach, J., Grube, M. (Eds.), Stress and symbiosis.

432 De los Rios, A., Perez-Ortega, S., Wierzchos, J., Ascaso, c., 2011. Differential effects of biocide
433 treatments on saxicolous communities. case study of the Segovia cathedral cloister (Spain). International
434 Biodeterioration and Biodegradation in press.

435 deCruz, A., Palmer, R.A., Culberson, C.F., Andreotti, A., Colombini, M.P., D., P., 2007. Preliminary
436 investigation of Er:YAG laser ablation for the removal of lichen from stone., In: Joyce H. Townsend, L.T.,
437 Francesca Cappitelli (eds), Conservation Science Archetype Publications London, p. 308.

438 deCruz, A., Wolbarsht, M.L., Andreotti, A., Colombini, M.P., Pinna, D., culberson, C.F., 2009.
439 Investigation of the Er:YAG laser at 2.94 mm to remove lichens growing on stone. Studies in conservation
440 54, 268-277.

441 deCruz, A., Wolbarsht, M.L., Palmer, R.A., Pierce, S.E., Adamkiewicz, E., 2005. Er:YAG laser
442 applications on marble and limestone sculptures with polychrome and patina surfaces, In: Physics, S.P.i.
443 (Ed.), Lasers in the Conservation of Artworks.pp. DOI: 10.1007/1003-1540-27176-27177_27114

444 Doehne, E.and Price, E. 2010. Stone Conservation: An Overview of Current Research, 2nd Edition
445 Institute, T.G.C. (Ed.), Los Angeles, CA.

446 European-Comission-Regulation, 2007. No 1451/2007. The second phase of the 10-year work program
447 referred to in Article 16(2) of Directive 98/8/EC of the European Parliament and of the Council
448 concerning the placing of biocidal products on the market. Official Journal of the European Journal 3-64.

449 Gomoiu, I., Radvan, R., Sarantopoulou, E., Cefalas, A.C., 2006. Lasers in biodeterioration, In: Schreiner,
450 M., Strlic, M. (Eds.), Handbook on the use of lasers in conservation and conservation science. COST
451 office G7, p. 20.

452 Goodis, H.E., White, J.M., Marshall, S.J., Marshall, G.W., 1993. Scanning electron microscopy
453 examination of intracanal wall dentin: hand versus laser treatment. Scanning Microscopy 7, 979-987.

454 Gueidan, C., Roux, C., Lutzoni, F., 2007. Using a multigene phylogenetic analysis to assess generic
455 delineation and character evolution in *Verrucariaceae* (Verrucariales, Ascomycota). Mycological
456 Research 111, 1145-1168.

457 Kushnir, E.T., A. Galun, M., 1978. "Oil Hyphae" of Endolithic Lichens and Their Fatty Acid
458 Composition. protoplasma 97, 47-60.

459 Leavengood, P., Twilley, J., Asmus, J.F., 2000. Lichen removal from Chinese Spirit path figures of
460 marble. Journal of Cultural Heritage, s71-s74.

461 López, A., Lamas, J., Ramil, A., Yáñez, A., Rivas, T., Silva, B., Taboada, J., 2010. Optimization of laser
462 cleaning parameters for the removal of biological black crusts in granites., In: R. Radvan, J.F.A., M.
463 Castillejo, P. Pouli, A. Nevin (Ed.), Laser in the conservation of Artworks VIII CRC Press, p. 227.

464 Manevitch, Z., Lev, D., Hochberg, M., Palhan, M., Lewis, A., Enk, C.D., 2010. Direct antifungal effect of
465 Femtosecond laser on *Trichophyton rubrum* onychomycosis. Photochemistry and Photobiology 86, 476-
466 479.

467 Marakis, G., Maravelaki, P., Zafiropulos, V., Klein, S., Hildenhagen, J., Dickmann, K., 2000.
468 Investigations on cleaning od black crusted sandstone using different UV-pulsed lasers. Journal of
469 Cultural Heritage 1, s61-s64.

470 Maravelaki-Kalaitzaki, P., Zafiropulos, V., Pouli, P., Anglos, D., Balas, C., Salimbeni, R., Siano, S., Pini,
471 R., 2003. Short free running Nd:YAG laser to clean different encrustations on Pentelic marble: procedure
472 and evaluation of the effects. Journal of Cultural Heritage 4, 77s-82s.

473 Nandakumar, K., Obika, H., Shinozaki, T., Ooie, T., Utsumi, A., Yano, T., 2002. Impact of pulsed
474 Nd:YAG laser irradiation on the growth and mortality of the biofilm forming marine bacterium
475 *Pseudoalteromonas carrageenovora*. Biofouling 18, 123-127.

476 Nandakumar, K., Obika, H., Utsumi, A., Ooie, T., Yano, T., 2004. In vitro laser ablation of laboratory
477 developed biofilms using an Nd:YAG laser of 532 nm wavelength. Biotechnology and Bioengineering 86,
478 729-736.

479 Nimis, P.L., Martellos, S., 2008. The Information System on Italian Lichens., In: Version 4.0. University
480 of Trieste, D.o.B., IN4.0/1. (Ed.).

481 Osherov, N., Yarden, O., 2010. The cell wall of filamentous fungi, In: Borkovich K.A. and D.J. Ebbole
482 (Ed.), Cellular and molecular biology of filamentous fungi. ASM Press, Washington D.C., pp. 224-237.

483 Oujja, M., Rebollar, E., Castillejo, M., Domingo, C., Cirujano, C., Guerra-Librero, F., 2005. Laser
484 cleaning of terracotta decorations of the portal of Palos of the Cathedral of Seville. Journal of cultural
485 heritage 6, 321-327.

486 Pinna, D., Salvadori, O., Tetriach, M., 1998. An anatomical investigation of calcicolous endolithic lichens
487 from the Triestre Karst (NE Italy). Plant Biosystems 132, 183-195.

488 Potgieter-Vermaak, S.S., Godoi, R.H.M., Grieken, R.V., Potgieter, J.H., Oujja, M., 2005. Micro-structural
489 characterization of black crust and laser cleaning of building stones by micro-Raman and SEM techniques.
490 Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy Volume 61, Issues , Pages 2460-
491 2467.

492 Pouli, P., Fotakis, C., Hermosin, B., Saiz-Jimenez, C., Domingo, C., Oujja, M., Castillejo, M., 2008. The
493 laser-induced discoloration of stonework; a comparative study on its origins and remedies. Spectrochimica
494 Acta Part A: Molecular and Biomolecular Spectroscopy, 932-945.

495 Pouli, P., Oujja, M., Castillejo, M., 2011. Practical issues in laser cleaning of stone and painted artefacts:
496 optimisation procedures and side effects Applied Physics A: Materials Science & Processing 1-18.

497 Sarantopoulou, E., Kollia, Z., Gomoiu, I., 2006. Preventing biological activity of *Ulocladium* sp spores in
498 artifacts using 157-nm laser. Applied Physics A 83, 663-668.

499 SCENIHR, 2009. Assessment of the antibiotic resistance effects of biocides. Scientific Committee on
500 Emerging and Newly Identified Health Risks, European Commission Health & Consumer Protection DG.,
501 Brussel, p. 87.

502 Scheerer, S., Ortega-Morales, O., Gaylarde, C., 2009. Chapter 5 Microbial deterioration of stone
503 monuments—An Updated Overview. 66, 97-139.

504 Schroeter, B., Sancho, L.G., Valladares, F., 1999. In situ comparison of daily photosynthetic activity
505 patterns of saxicolous lichens and mosses in Sierra de Guadarrama, Central Spain. The Bryologist 102,
506 623-633.

507 Siano, S., 2007. Principles of laser cleaning in conservation., In: G7, C.a. (Ed.), Handbook on the use of
508 laser in conservation and conservation science.

509 Smijs, T.G.M., Schuitmaker, H.J., 2003. Photodynamic inactivation of the dermatophyte *Trichophyton*
510 *rubrum*. Photochemistry and Photobiology, 5.

511 Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., Gilbert, O.L., James, P.W., Wolseley, P.A., 2010.
512 The lichens of Great Britain and Ireland. British Lichen Society London.

513 Speranza, M., Sanz, M., Oujja, M., de los Ríos, A., Pérez-Ortega, S., Ascaso, C., Castillejo, M., 2011.
514 Laser cleaning investigation to remove biological crust on stone. SPIE Optical Metrology Conference
515 O3A: Optics for Arts, Architecture and Archeology III Technical Summaries Munich, Germany.

516 Tornari, V., Fotakis, C., Georgiou, S., Zafiropulos, V., Anglos, D., 2006. Laser cleaning of encrustation,
517 In: Fotakis, C., Georgiou, S., V., Z., Tornari, V. (Eds.), Laser in the preservation of cultural heritage
518 principles and applications. Taylor & Francis, p. 336.

519 Tretiach, M., Bertuzzi, S., Salvadori, O., 2010. Chlorophyll a fluorescence as a practical tool for checking
520 the effects of biocide treatments on endolithic lichens. International Biodeterioration and Biodegradation
521 64, 452-460.

522 Tretiach, M., Crisafulli, P., Imai, N., Kashiwadani, H., Heemoon, K., Wada, H., Salvadori, O., 2007.
523 Efficacy of a biocide tested on selected lichens and its effects on their substrata. International
524 Biodeterioration and Biodegradation 59, 44-54.

525 Vural, E., Winfield, H.L., Shingleton, A.W., Horn, T.D., Shafirstein, G., 2007. The effects of laser
526 irradiation on *Trichophyton rubrum* growth. Lasers in Medical Science 23, 349-353.

527 Wierzchos, J., Ascaso, C., 1994. Application of back-scattered electron imaging to the study of the lichen-
528 rock interface. Journal of Microscopy 175, 54-59.

529 Wierzchos, J., Ascaso, C., 1996. Morphological and chemical features of bioweathered granitic biotite
530 induced by lichen activity. Clays and Clay Minerals 44, 652-657.

531 Wierzchos, J., De los Ríos, A., Sancho, L.G., Ascaso, C., 2004. Viability of endolithic microorganisms in
532 rocks from the McMurdo Dry Valleys of Antarctica established by confocal and fluorescence microscopy.
533 Journal of Microscopy 216, 57-61.

534 **Figure legends**

535 **Figure 1.** Crustose thallus of *V. nigrescens* (white arrows in A and B) and areoles immersed
536 perithecia and their ostioli (arrows in B). Laser cleaned areas (asterisks in A and D) with lichen

537 thallus remains (black arrows in A, C-E). A and C: stereomicroscopy images; B and D: SEM-SE
538 images; E: LTSEM image.

539 **Figure 2.** Micro-Raman spectra of the control dolostone sample (A), and stone surface colonized
540 by *Verrucaria nigrescens* before (C) and after laser irradiation (B).

541 **Figure 3.** SEM-BSE images of *V. nigrescens* epilithic thalli and endolithic colonization of
542 dolomite stone before (A-D) and after laser treatment (E-F).

543 Upper cortex (thin white arrows in A and C), photobiont layer and mycobiont hyphae (white
544 asterisks in A and C), fungal cells with several lipid globules (black arrows in B) and algae
545 (white open arrows in B), and lichen medullar region (thin black arrows in A and C) from a
546 control *V. nigrescens* thalli. Strong signals produced by the OsO_4 staining of cytoplasmatic lipids
547 in fungal hyphae from vital lichen thalli (box in C) and in clusters of fungi localized in endolithic
548 zones (D). Empty spaces in the dolomite surface after laser removal of lichen thalli (black
549 asterisks in E). Microorganisms localized in endolithic zones near the surface and lichen medullar
550 region were completely destroyed (thin black arrows). Other zones showed fungal cells with a
551 high degree of plasmolysis (white boxes in E and detail in F).

552 **Figure 4.** SEM-BSE images of fungal epilithic and endolithic colonization of dolomite stone
553 before (A) and after (B-D) laser treatment. Free fungi (black arrows and upper box in A) and
554 endolithic algal and fungal cells (white thin arrow and lower box in A) in control sample.
555 Endolithic clusters near the stone surface damaged by the laser heat effect (B). Clusters of
556 phototrophic and heterotrophic microorganisms (white thin arrow and box in C, and white thin
557 arrows in D) from deeper stone zones (until 1mm) showed a weak laser effect. Empty spaces in
558 the dolomite surface after laser removal of lichen thalli (black asterisks). fc: fungal cell; ac: algal
559 cell.

560 **Figure 5.** SEM-BSE (A-D) and LTSEM (E-F) images of lichen thalli and microorganisms
561 localized near the laser cleaned areas. Lichen thalli next to the irradiated surface (white arrows in
562 A) with empty algal and fungal cells that only conserved their walls (white asterisks in A).
563 Fungal and algal cell clusters affected by the thermal laser (black thin arrows and box in B).
564 Thalli from an intermediate localization (white arrows in B) showed evident fungal cell wall and

lipid body damage (detail in C) compared with control thalli (D). *V. nigresecens* hidrated control thalli (E) showing algal cells (ac) and chloroplast (c) (white open arrows and box in E). The complet laser destruction of algal and fungal cells can be observed (F) with some fungal lipid body debris (black empty arrows).

Fig. 6. Fluorescence microscopy 3D reconstruction MIP images of SYTOX Green stained lichen symbiont cells from control lichen thalli (A) and from lichen thalli located in neighbor laser cleaned zones (B). Image A reveals red autofluorescence signal of algal (a) cells and green autofluorescence of fungal cells (f). Image B show red autofluorescence signal of algal (a) cells and green signal of nuclei of algal cells with damaged membranes (arrows).

Table 1. List of the bands and the corresponding assignments determined by micro-Raman spectroscopy in the dolomite and *Verrucaria nigrenscens* thalli.

Wavenumbers (cm^{-1})	**Vibrational assignment**	**Origin of bands**
1744	ν (C=O)	Hydrocarbons
1615	ν_a (C-O)	Hydrocarbons
1580	Graphitic carbon	Organic compounds
1440	ν_s (C-O) + ν (C-C)	Hydrocarbons
1340	Disordered carbon	Organic compounds
1097	Symmetric stretching of (CO_3^{2-})	$CaCO_3$, dolomite
727	In-plane bending of (CO_3^{2-})	$CaCO_3$, dolomite
299	Lattice vibration of (CO_3^{2-})	$CaCO_3$, dolomite
175	Lattice vibration of (CO_3^{2-})	$CaCO_3$, dolomite

Figure

Figure 1.

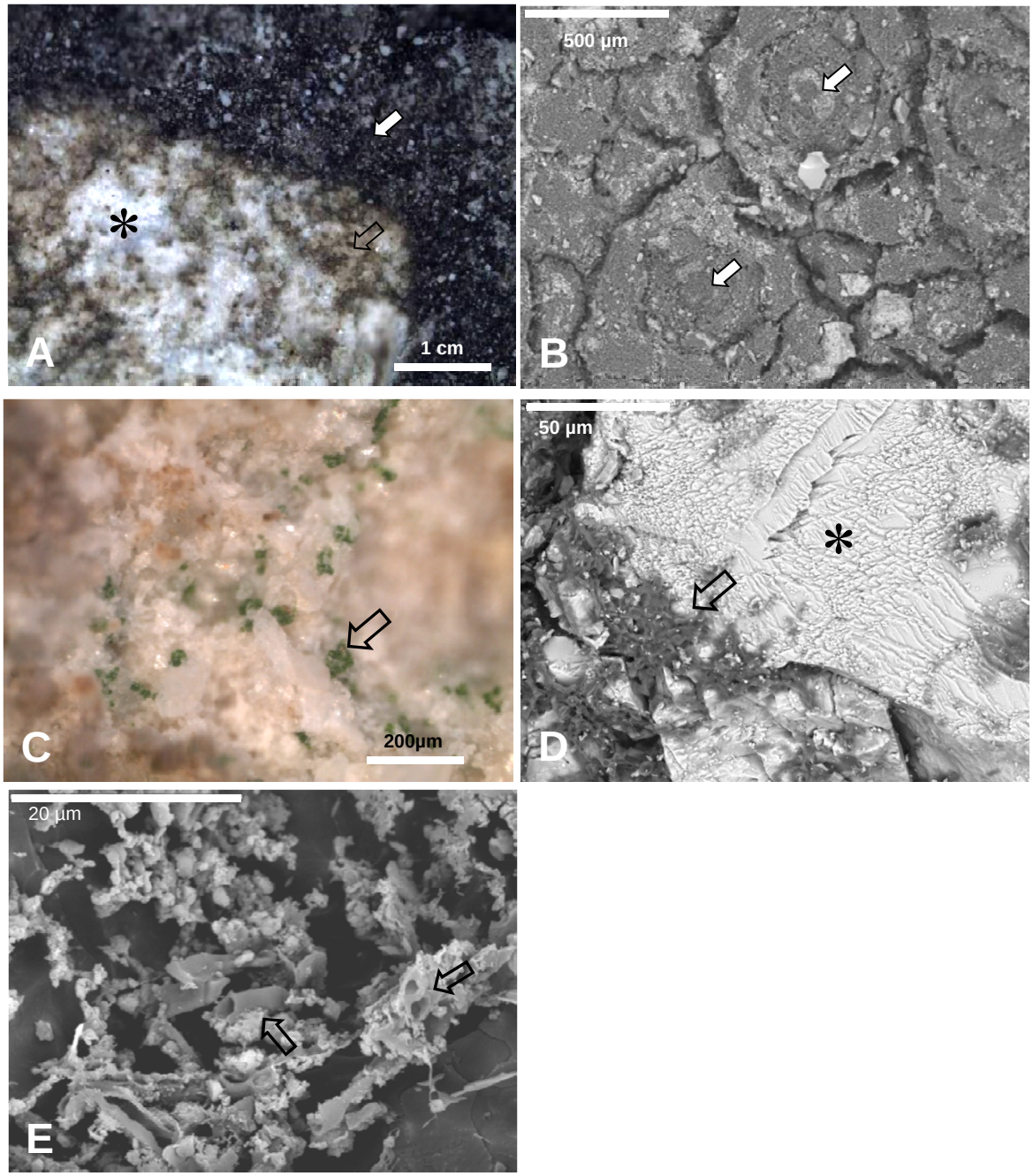

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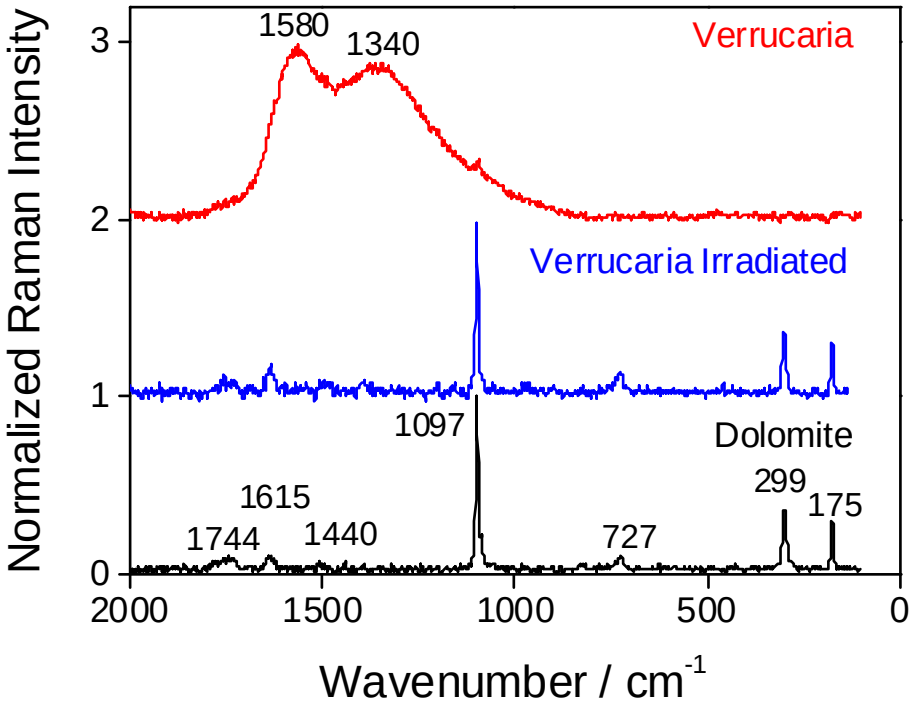
Figure

Figure 2.



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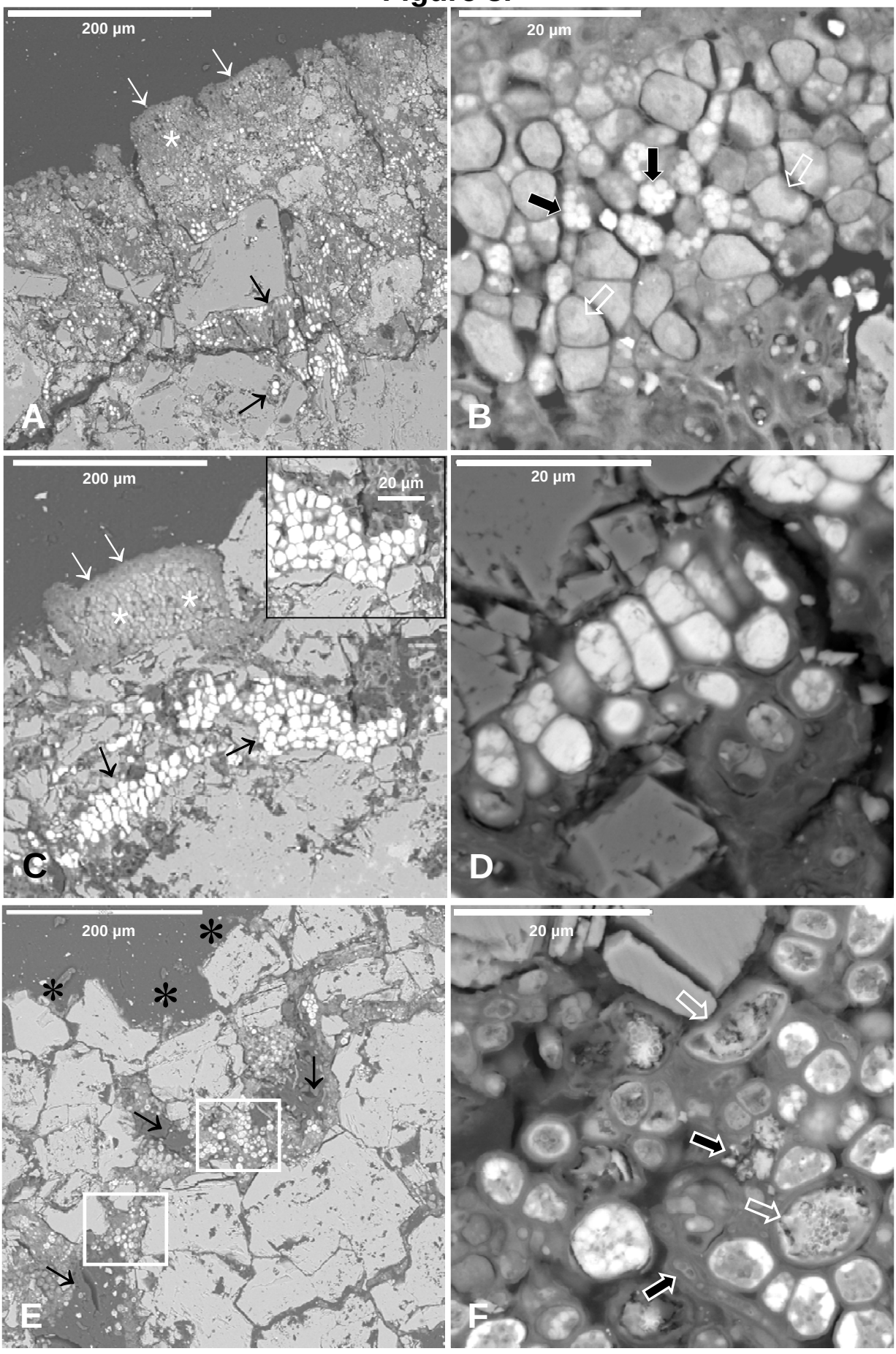

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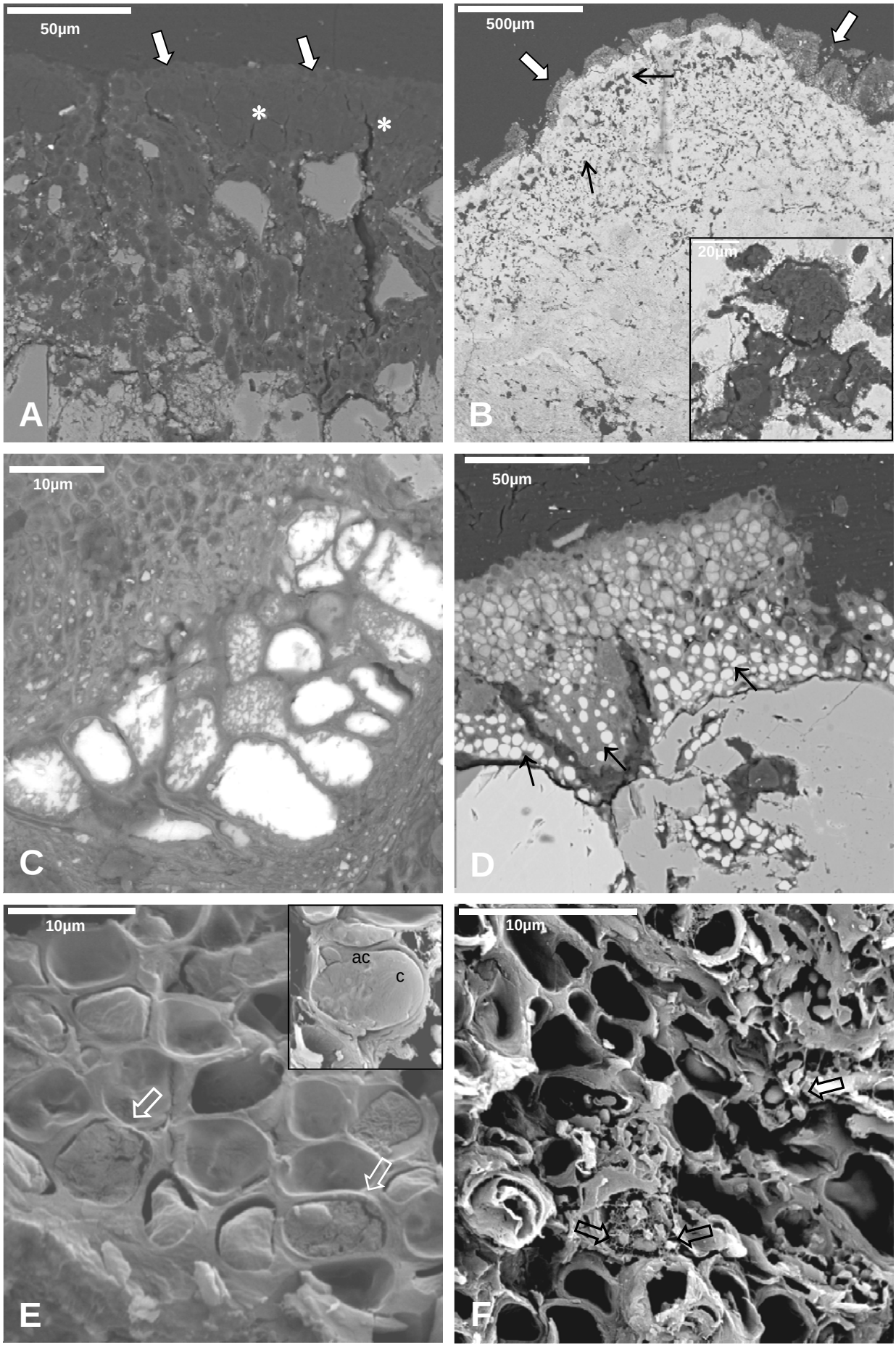

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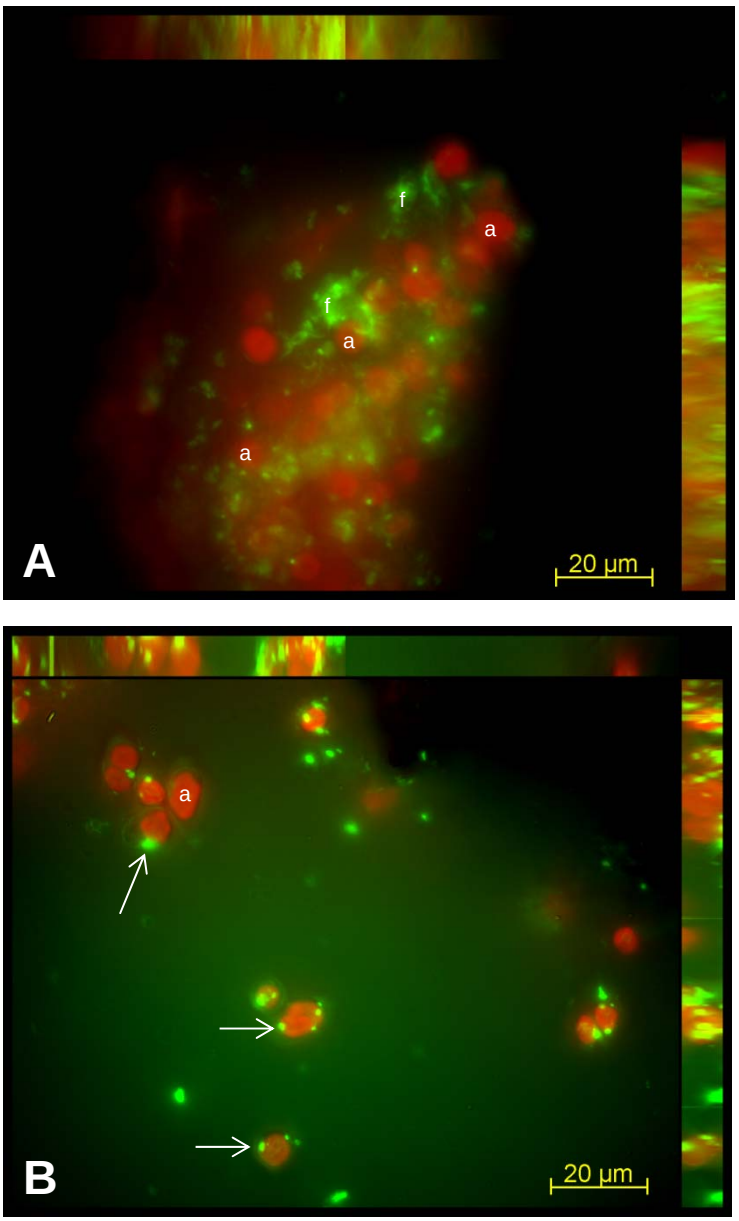


Madrid, December 19, 2011.

Dear IBB-Editor: Dr. Christine Gaylarde

Please find enclosed our manuscript to be considered for publication in the IBBS15 special issue
Title:ND:YAG laser irradiation effects on epilithic and endolithic microorganisms

Authors: M. Speranza, M. Sanz, M. Oujja, A. de los Ríos, J. Wierzchos, S. Pérez- Ortega, M. Castillejo and C. Ascaso

The aim of the present study was to investigate whether endolithic and epilithic microorganisms are damaged at structural and physiological level by laser irradiation.

Considering, the limitations of traditional methods to control lithic microorganisms and the inefficacy to control the endolithic microorganisms involved in internal physical and chemical stone damage, we decide to evaluate laser irradiation as an alternative.

Laser is widely used in many countries to remove blacks crusts in monumental stone and, sometimes, also in natural stone. Furthermore, what happens with endolithic and epilithic microorganisms after the application of a laser treatment remains completely unknown in most cases.
In relation with the advantages of the experimental design and microorganism laser-damage evaluation:

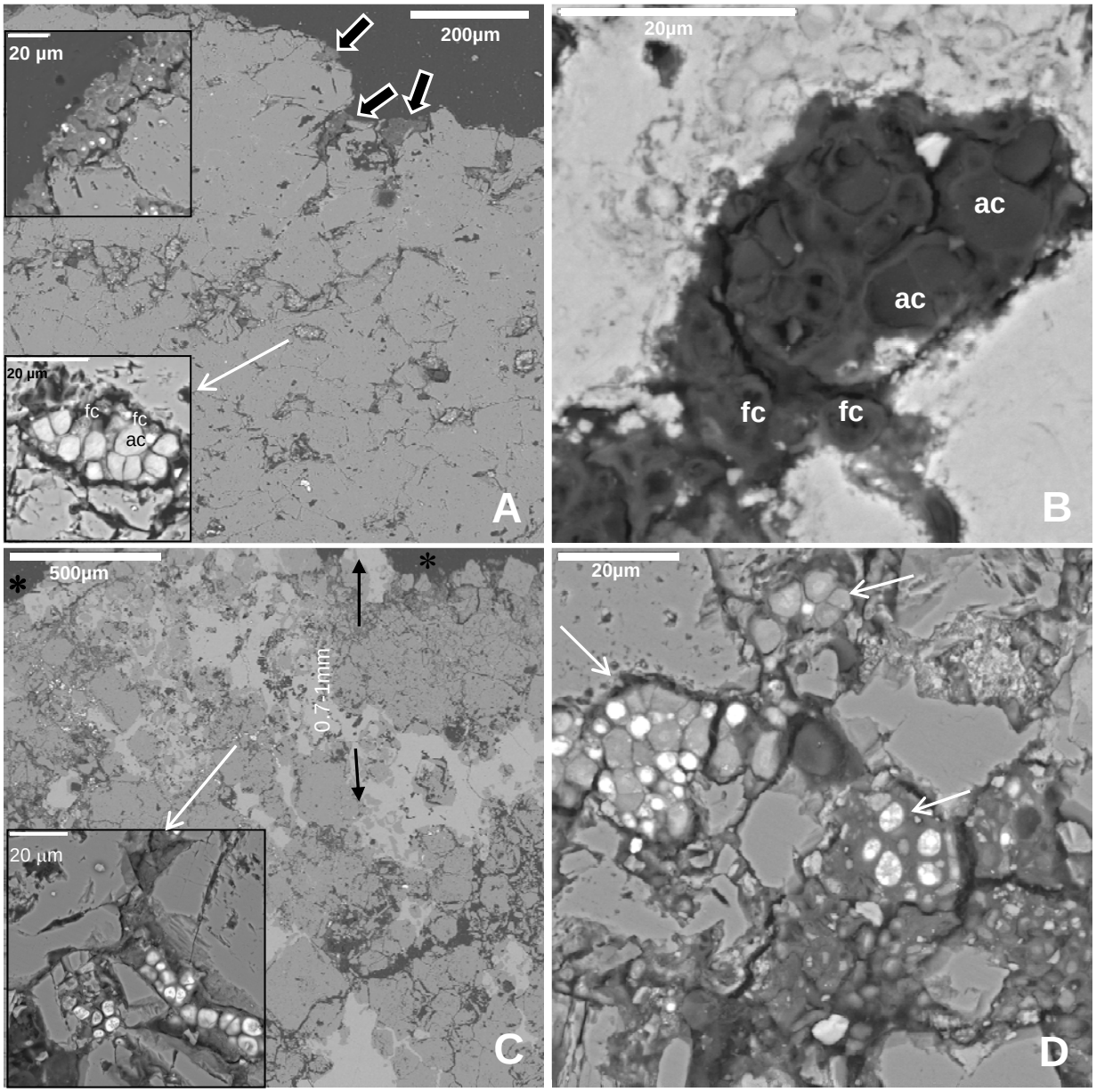
We understand that the effect of laser as an altering agent of lithic microecosystems must be tested on non-disturbed microecosystems, instead of being tested on plate cultures or through any other method commonly used by microbiologists, as these cultures are not the real microorganisms we are working on. These experiments allowed us showing *in situ* for the first time that lithic microorganisms several micra inside the stone undergo severe thermal damage caused by laser when applied on the stone surface to remove epilithic microorganisms.
Furthermore, the present results indeed contribute the first knowledge on the sensitivity of endolithic and epilithic microorganisms to laser irradiations according to microscopic and physiologic studies.
Therefore, laser is a new environmentally-friendly tool to control the growth of endolithic microorganisms and a way to reduce biodeterioration in stone materials**.**

My co-authors and I look forward to hearing from you soon.
Yours sincerely,

Dr. Mariela Speranza

Figure

Figure 4.



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