Biofilm formation on natural stones exposed to urban atmosphere: evaluation of biosusceptibility of different lithotypes

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1. Introduction

Outdoor stone substrates of the architectural heritage represent a challenging habitat for biological growths. The intense fluctuation of the environmental factors (i.e. temperature and relative humidity), the exposure to intense solar radiation and wind, low availability of nutrients and pollutants concentrations determine particularly stressful conditions (Gorbushina, 2007). Nevertheless, biological colonisation on heritage structure is ubiquitous and has been recognized as a major mechanism of alteration of stone substrates (May, 2010, Siegesmund and Snethlage, 2011, Palla and Barresi, 2017).

Bioreceptivity of stone is influenced by the inherent mineralogical and chemical composition of the substrate (Miller et al., 2012). Physical properties, in particular porosity and water absorption, are also relevant factors. In urban polluted environment, chemical and mechanical deterioration of the stone substrate influence biofilm formation (secondary bioreceptivity). For instance, (Prieto and Silva, 2005) and (Vazquez-Nion et al., 2018) highlighted the increased colonisation of granite at the increased roughness and porosity of the surface. The conservation history also plays a significant role in promoting colonisation, as the presence of altered restoration treatments can provide source of energy for microbial proliferation (Cappitelli et al., 2007).

The most diffused effect of biofilm development is an aesthetical alteration of the surfaces, with formation of coloured patina (generally dark or green) that clearly reveals the presence of biocolonization by means of simple visual observations. The aesthetical change of the surfaces is of primary concern with respect to the preservation of cultural heritage. The perceived value and appreciation of stone façade is strongly influenced by the presence of dark and coloured patina and, in general, by soiling. Although deposition of particulate matter, soil dust and other solid pollutants is the main responsible for such phenomenon in urban environment, biofilm formation can also provide a significant contribution (Gorbushina et al., 1993, Grossi et al., 2003). (Brimblecombe and Grossi, 2005) proposed thresholds of tolerance above which the extent of aesthetic alteration become publicly unacceptable and the pressure for cleaning interventions may rise as a result. Within the decision-making process involving preservation authorities, heritage managers and conservators, aesthetic alteration due to biological colonization can therefore be included among the parameters driving to the necessity of intervention.

Subaerial biofilm on stone is a complex surface-associated community embedded in a self-produced polymeric matrix (EPS) enabling microorganisms to develop coordinated and efficient survival strategies. It is made of fungi, algae, cyanobacteria and heterotrophic bacteria. The biological succession on stone surfaces and the process of formation of biofilm has been summarized by (Warscheid and Braams, 2000), who also point out the possible deterioration effects associated to the different stages of colonization. Such effects are the result of the living activity of the biofilm community, which exploits chemical, physical-mechanical mechanisms able to modify the stone microenvironment and support its growth (Warscheid and Braams, 2000, Villa et al., 2016). Chemical mechanisms are mainly related to biocorrosion process, occurring as a result of the biotic production of inorganic and organic acidic species which can weaken the mineral matrix of the stone according to different processes (McNamara and Mitchell, 2005). Various deterioration patterns ranging from localized pitting to scaling and detachments of stone fragments have been associated to such mechanisms (Sterflinger and Piñar, 2013, Cuezva et al., 2012). The production of EPS and subsequent desiccation/hydration cycles can cause mechanical stresses on the mineral structure (Guiamet et al., 2013). Mechanical stress is also induced by hyphae and filamentous growth that penetrate into the stone substrate (Hoffland et al., 2004, Sterflinger and Krumbein, 1997). Such stress can lead to granular disaggregation of the mineral matrix and promote physical damage to the stone, i.e. secondary porosity formation and increased water absorption (Morando et al., 2017).

Biocolonization is not limited to the surfaces directly exposed to the environment. Endolithic microorganisms can penetrate up to several millimetres in depth within the crystalline matrix and porosity of the stone, sometimes causing damages to the stone (Siegesmund and Snethlage, 2011, Caneva et al., 2014). However, even if microbial growth can lead to biodeterioration, the presence of subaerial biofilms and the identification of specific microorganisms with known reactivity towards the stone substrate does not necessarily imply that a biodeterioration process is in progress (Villa et al., 2016). Moreover, in specific conditions, biofilms have been associated to increased resistance of the stone surfaces, and bioprotection effects have been highlighted as a result of colonization (Mottershead et al., 2003, Carter and Viles, 2005, Morando et al., 2017).

Microbial abatement is commonly achieved using biocides. It is well known that the use of biocides has detrimental effects on environment and health. The possible interaction between biocide and stone has also to be tested in advance in order to assure the non-harmfulness of the treatment towards the substrate. Before deciding if the removal of microbial colonization is overall beneficial for stone conservation, the impacts of microbial growth with deterioration induced by physico-chemical processes at the same site should be compared. Additionally, it is well known that in outdoor conditions once structures have been cleaned, recolonization is inevitable and may come shortly after treatment (Giacomucci et al., 2011, Cappitelli et al., 2012). A reason is that the presence of dead cells, biofilm matrix polymers and biocidal components, especially in endolithic zones not subjected to washing away by rainfall, can provide a source of nutrients for the development of new lithobiontic microbiota (de los Ríos et al., 2012). In many cases well-defined differences of the microbial community between the untreated and the treated surfaces are seen (Hallmann et al., 2013) and subsequent recolonization might be more aggressive or disfiguring than the eradicated one (Warscheid and Leisen, 2011).

In the present work, we studied the extensive biological colonization affecting the stone surface of the Cathedral of Monza (Italy) (Fig. 1~~Fig. 1~~). In particular, four metamorphic stone types of the façade showing different compositional, mineralogical and microstructural features are considered: Musso, Crevoladossola and Candoglia marble, together with Oira stone. The façade is a remarkable example of 15th century architecture, which underwent several major interventions between 19th and 20th century (Cassanelli, 1988). It is characterized by alternated dark and white-coloured rows of stone blocks composing the cladding and by the use of a wide range of stone materials for both construction and decorative purposes. The current state of conservation is affected by dark and green biofilms irregularly covering the marble elements of the top levels of the façade. Flat blocks and frames of Oira stone, on the other hand, do not show macroscopic evidence of colonization.

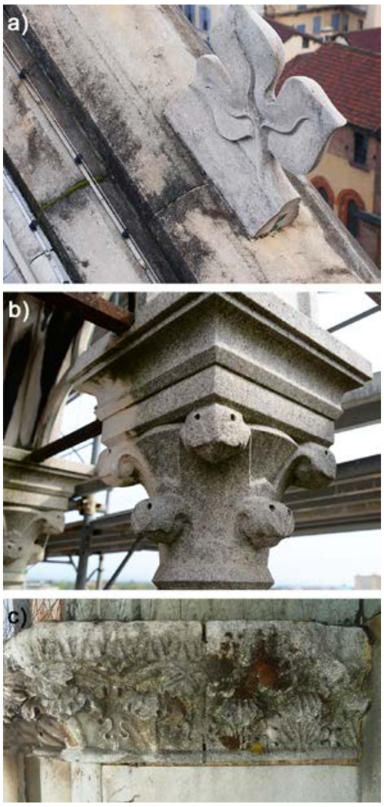


Fig. 1 - Extensive biocolonization on the stone surfaces of the Monza Cathedral: black biofilms on top elements and moss growth within mortar joints (a); diffused green biofilms irregularly covering Candoglia capitals (b); biological growth on sculpted decorative elements (c)

The main objectives of the present research are as follows:

1. to evaluate the susceptibility of the different lithotypes exposed to similar environmental conditions to biocolonization;
2. to evaluate the deterioration effects associated to biofilm formation vs. chemical-physical damage in order to assess the necessity/suitability of a biocidal treatment;

2. Materials and methods

Stone characterization and evaluation of the state of conservation

The preliminary on-site investigation of the stone surfaces was made by means of a portable digital microscope Dino-Lite Premiere AM7013MT, colour CMOS sensor and white LED illuminator on not-colonized areas and on area with increasing extent of epilithic growth.

Micro-samples for the laboratory analysis were collected by scalpel and chisel according to the standard guidelines (Uni, 2012) from the top levels and spires of the façade, where biocolonization is mostly concentrated. For each lithotype, samples were collected from areas showing macroscopic evidences of biological colonization and from reference adjacent areas with similar exposure conditions but no biofilm. Samples were immediately stored in polyethylene containers and transferred to the lab. Polished cross-sections were prepare by embedding stone fragments in bi-component epoxy resin (Mecaprex MA2+, Presi).

Micro-samples were studied by means of a Leica M250C stereomicroscope, integrated with a Leica DFC290 digital camera for image capture. Polished cross-section were observed by means of a Leica DMRE optical microscope in dark-field mode, equipped with a colour digital camera Nikon D750.

The mineralogical composition of the stones was assessed on powdered samples by a Philips PW1830 X-ray diffractometer (XRD) using Cu Kα radiation (λ = 1,54058 Å), PW3020 generator and Bragg-Brentano geometry.

The compositional features of stones were investigated by Fourier Transformed Infrared Spectroscopy (FTIR), using a Thermo Nicolet 6700 FTIR spectrometer in KBr dispersion with a DTGS detector in the spectral range 4000-400 cm^{-1}. Epilithic biofilms from stone surfaces were sampled after mechanical separation from the mineral matrix of the substrates and analysed in compression diamond cell by means of a Thermo Nicolet Continμm FTIR microscope with a MCT detector in the spectral range 4000-600 cm^{-1}.

Environmental Scanning Electron Microscopy (ESEM) and Energy Dispersive X-ray (EDX) analyses, using Zeiss EVO 50 EP ESEM, equipped with an Oxford INCA 200 - Pentafet LZ4 spectrometer were performed on samples' fragments and polished cross sections.

Visualization of biofilm structure

Biofilm samples were collected using the non-invasive method of adhesive tape strips. Strips were gently applied to the stone surface and were then placed on sterile glass microscope slides and kept in a box until arrival in laboratory. The adhesive tape strips were immediately analyzed by microscopy.

The structure and the architecture of biofilms growing on different lithic surfaces were investigated by CLSM in both fluorescence and reflection modes as previously reported by Villa et al. (Villa et al., 2015). The reflection mode (excitation at 488 nm, and emission at 480 to 490 nm) allowed recording of reflective signals originating from inorganic solid material. Autofluorescence from photosynthetic pigments was viewed in the red channel using the 633 nm line of an Ar/HeNe laser in the emission range of 650 to 750 nm. Extracellular polysaccharides (EPS) were labeled with the lectin Concanavalin-A- Texas red (Molecular Probes, Inc., Eugene, OR, USA) (0.8 mM final concentration) and observed in the red channel (excitation at 561 nm line, and emission at 590 to 630 nm).

The FilmTracer™ calcein green biofilm stain (Thermo Fisher Scientific, Italy) was used to visualize live cells, according to the manufacturer's instructions. Indeed, the stain is an esterase substrate (Calcein AM) that is non-fluorescent until non-specific esterases hydrolyze it to a green-fluorecent calcein. Signal from esterase-positive cells was captured in the green channel using an Argon gas laser, with excitation at 488 nm and emission range of 500 to 550 nm.

Captured images were analyzed with the software Imaris (Bitplane Scientific Software, Switzerland) for 3D reconstructions of biofilm samples. Living phototrophic and the heterotrophic microorganisms were distinguished by coupling the results from FilmTracer™ calcein green biofilm stain staining with those obtained from pigment autofluorescence signals. The biovolumes and the mean thickness of biofilms were calculated using PHLIP, a freely available Matlab-based image analysis toolbox (http://phlip.sourceforge.net/phlip-ml). The biovolume represented the overall volume of a biofilm (µm3) and could be used to estimate its total biomass. It was defined as the number of foreground pixels in an image stack multiplied by the voxel volume, which is the product of the squared pixel size and the scanning step size (Mueller et al., 2006). The thickness parameter is widely used to describe the morphology of the biofilms. The function first applies a height projection transformation to the image stack where for every point in the xy plane the maximal height h of the corresponding foreground pixels in z direction is stored. The average of the resulting distribution of pixel height (h) is then calculated and represents the mean thickness (Xavier et al., 2003).

The emission spectra of photosynthetic pigments were obtained using a wavelength λ-scan function of the CLSM as reported by Roldan et al. (Roldán et al., 2014). Region of interest (ROIs) representing single cells were used to obtain fluorescence spectra. The fluorescence spectra were analyzed by Pickfit deconvolution software (PeakFit, SPSS, Inc.) to resolve individual phycobiliproteins as reported by Wolf and Schüβler (Wolf and SchÜBLer, 2005). Representative fluorescence spectra of dehydrated and rewetted cells within the biofilm are presented.

3. Results and discussions

Stone characterization

The main mineralogical features of the lithotypes from the façade are summarized in Tab. 1~~Tab. 1~~. Candoglia and Musso are calcitic marbles showing medium to medium-to-coarse grain size, respectively. The main mineralogical phase is calcite in both stones, with quartz as accessory mineral. Pyrite can also be found in Candoglia. These two lithotypes can be considered quite similar form the mineralogical and petrographical point of view. Crevola is a dolomitic marble with a medium-to-fine grain structure. The main mineralogical phase is dolomite, with the distinctive presence of minor phlogopite (trioctahedral magnesium mica belonging to the biotite series) in form of thin isolated crystals tightly packed within the dolomite grains. Oira stone has a rather different mineralogical composition with respect to all the other marbles employed in the façade. It is a very compact meta-peridotite deriving from the serpentinization of ultramafic rocks. Its composition includes lizardite as main mineralogical phase, together with clinochlore and minor magnesioferrite. It has been introduced in the building during a restoration of the late XIXth cent. In order to replace another carbonatic lithotype that was highly deteriorated, Varenna stone.

Tab. 1 - Mineralogical composition of the stones by means of XRD analysis

	Calcite	Dolomite	Quartz	Mica	Lizardite	Clinochlore	Magnesioferrite
Candoglia	+++	-	+	-	-	-	-
Musso	+++	-	+	-	-	-	-
Crevola	-	+++	-	+	-	-	-
Oira	-	-	-	-	+++	++	+

The different compositional features of the stones are highlighted by FTIR results (Fig. 2~~Fig. 2~~). The three marbles show similar FTIR patterns, with major absorptions in the 1400 cm^{-1} region, resulting from the stretching vibrations of the carbonate bond (CO_3^{2-}). This is associated to the presence of calcite in Candoglia and Musso marble, as indicated by the characteristic absorption peaks at 1420, 875 and 713 cm^{-1}. The dolomite content of Crevola marble determines a slight shift of the related absorption peaks to higher wavenumbers with respect to the previous ones, which can be detected in this case at 1435, 880, 730 cm^{-1}. The low-intense peak at 1100 cm^{-1} is due to the Si-O stretching vibration of phlogopite. Oira stone is characterized by a silicate-based composition with major absorption at 1088 and 948 cm^{-1} due to the stretching vibrations of the Si-O bond (Gulotta et al., 2017). The strong absorptions around 3400 cm^{-1} and the sharp peak at 3689 cm^{-1} are due to the stretching vibrations of hydroxyl groups typical of lizardite (Trittschack et al., 2012). As far as the general state of conservation is concerned, it can be noticed that all marbles shows signs of chemical alteration. The broad peaks in the 3400-3500 cm^{-1} region, the shoulders at 1620 cm^{-1}, and the low-intense peaks at 1150-1115 cm^{-1}, particularly evident in the spectra of Musso and Candoglia, can be related to the presence of gypsum. The prolonged exposition to outdoor polluted environmental conditions promotes sulphation processes of the calcitic and dolomitic substrates, according to well-known mechanisms (Malaga-Starzec et al., 2004, Watt et al., 2009).

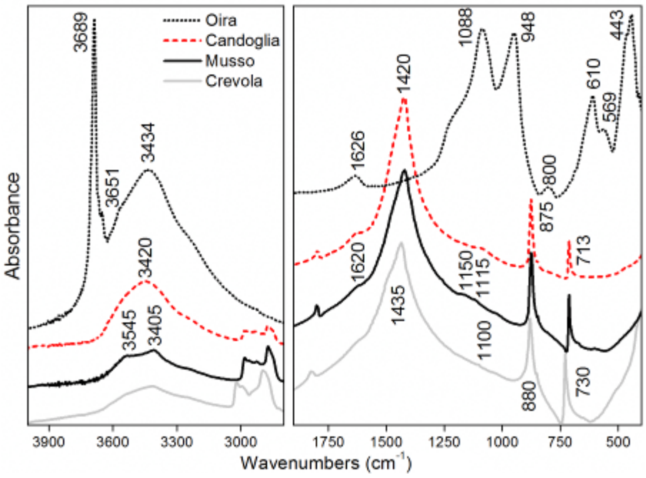


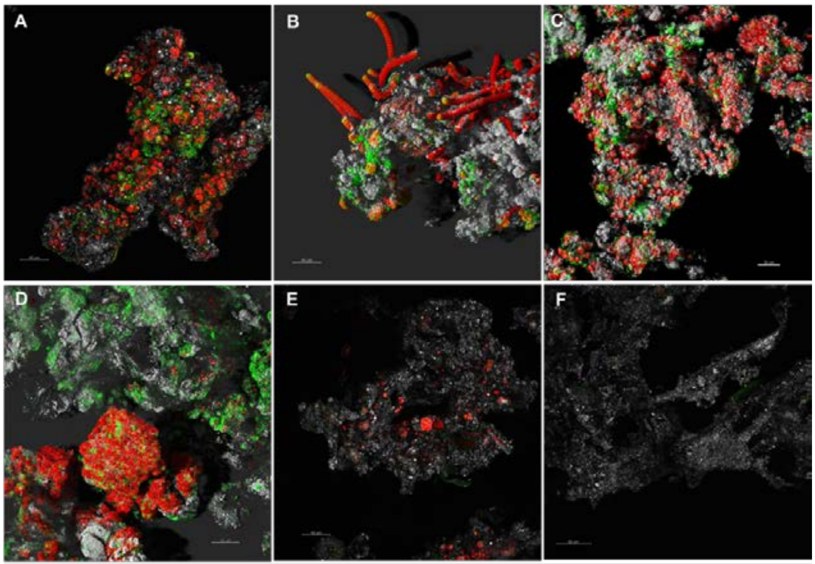
Fig. 2 - FTIR compositional characterization of the Candoglia, Crevola and Musso marble and Oira stone

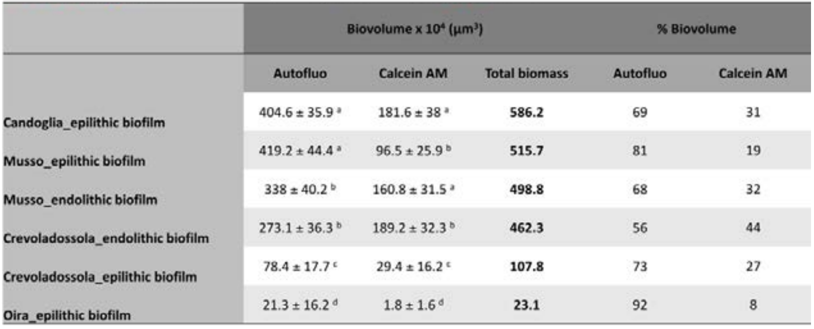
Biofilm characterization

Representative biofilm structures observed for different lithic surfaces are presented in Fig. 3~~Fig. 3~~. The images correspond to three-dimensional blend reconstructions obtained from confocal images series with the dedicated IMARIS software, including virtual shadow projections on the right-hand side to represent biofilm sections. The associated biovolumes and mean thickness calculated from the series of images are also provided.

Overall, we observed the predominance of phototrophic communities in all the specimens analyzed, while the Calcein stain did not show an extended heterotrophic bacterial community. However, these communities were highly developed in zones having weak or absent photosynthetic pigment fluorescence.

According to the biovolumes analysis the following colonization rate were established: Candoglia marble > Musso marble (epilithic = endolithic) > Crevoladossola marble (endolithic>epilithic) > Oira stone. The biofilm colonizing Candoglia marble was mainly composed of one cyanobacterial morphotype, surrounded by a sheath of capsular-like extracellular polysaccharidic substances Fig. 4~~Fig. 4~~. The space around the aggregates of coccoid cyanobacteria was filled with multicellular clusters of heterotrophic bacteria. The heterotrophs were frequently observed attached to the outer sheaths of cyanobacterial aggregates.





	Biovolume x 10⁴ (µm³)			% Biovolume	
	Autofluo	Calcein AM	Total biomass	Autofluo	Calcein AM
Candoglia_epilithic biofilm	404.6 ± 35.9 ^{a}	181.6 ± 38 ^{a}	**586.2**	69	31
Musso_epilithic biofilm	419.2 ± 44.4 ^{a}	96.5 ± 25.9 ^{b}	**515.7**	81	19
Musso_endolithic biofilm	338 ± 40.2 ^{b}	160.8 ± 31.5 ^{a}	**498.8**	68	32
Crevoladossola_endolithic biofilm	273.1 ± 36.3 ^{b}	189.2 ± 32.3 ^{b}	**462.3**	56	44
Crevoladossola_epilithic biofilm	78.4 ± 17.7 ^{c}	29.4 ± 16.2 ^{c}	**107.8**	73	27
Oira_epilithic biofilm	21.3 ± 16.2 ^{d}	1.8 ± 1.6 ^{d}	**23.1**	92	8

Fig. 3 – Confocal laser scanning imaging (photos) and related properties (table) of biofilms growing on Candoglia marble (panel A), Musso (panels B-epilithic biofilm, C-endolithic biofilm), Crevoladossola (panels D-endolithic biofilm, E-epilithic biofilm) and Oira stone (panel F). Colour key: phototrophs, red (autofluorescence); heterotrophs, green (calcein AM); stone, grey (reflection). Data in the Table represent the means + the SD of independent measurements. Letters provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P< 0.05), means sharing the same letter are not significantly different from each other

The epilithic biofilm on Musso was characterized by several morphotypes of phototrophic microorganisms. It was possible to recognize coccoid and rod-shaped structures assembled in clusters and protruding septated filaments. Interestingly, the network-forming filaments appeared to be calcified. Ones more, aggregates of heterotrophic bacteria were found between the phototrophic assemblages. The most characteristic feature of the endolithic growth in Musso samples was the presence of a stratified biofilm composed of a thin and compact bottom layer of heterotrophs and a discontinuous upper layer for active coccoid phototrophs. A similar architecture was observed for endolithic biofilms of Crevoladossola. By contrast, the surface of the lithotypes Crevoladossola was poorly colonized by a cyanobacterial-rich biofilm dominated by only one coccoid morphotype organized in small assemblages.

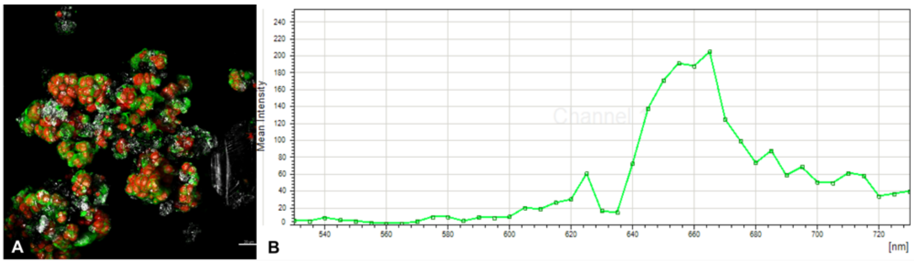


Fig. 4 – Panel A shows a coccoidal cyanobacterial morphotypes (red signal, autofluorescence) retrieved on Candoglia marble surrounded by a sheath of capsular-like extracellular polysaccharidic substances (green signal, Concanavalin A). Panel B presents the emission spectra recorded from coccoid cyanobacteria inhabiting Candoglia marble. Spectral profile shows individual phycobiliproteins: Phycoerythrin, 575 nm; Phycocyanin, 645 nm; Allophycocyanin, 665 nm; Chlorophyll a, 685 nm (Wolf and SchÜBLer, 2005).

Despite the quali- and quantitative differences on biofilm coverage and composition, some common features were observed in all samples analyzed. The lectin-binding analysis combined with CLSM revealed the presence of extracellular polymeric substances glycoconjugates (i.e. polysaccharides, including those ones covalently linked to proteins and/or lipids) in all epilithic and endolithic biofilms colonizing the different lithotypes. Two major structural domains were observed in the biofilms: i) EPS glycoconjugates clearly associated with the sheaths of phototrophic organisms, and ii) network-like EPS glycoconjugates with detectable as extended sheet-like structures. Furthermore, it was possible to observe that both the photosynthetic and the heterotrophic components of the biofilms retrieved on different lithotypes were metabolically active. All the morphotypes of phototrophic microorganisms observed on the different substrata showed autofluorescence emission signals within the red range in response to 488-nm laser excitation. Most of the photosynthetic cells exhibited characteristic emission peaks between 645-685 nm, corresponding to phycobiliproteins and chlorophyll *a*. Only the filamentous phototrophs presented a marked emission peak at 575 nm, attributable to the presence of phycoerythrin. We did not observe changes in the emission spectra of the same morphotypes coming from different substrata. Thus, according to Roldan et al. (Roldán et al., 2014), these cells were classified as viable and healthy. In the same way, the intense green signals coming from the heterotrophic component of the biofilm communities indicated the predominance of live cells. In metabolically active cells, Calcein AM is converted by cytosolic esterases into green fluorescent Calcein (Sirenko et al., 2015). The fluorescent Calcein is retained by live cells with intact membranes (Bridier et al., 2011).

Overall, the microbiological investigations revealed that: i) Phototrophs comprised the largest proportion of biofilm communities in all the specimens analyzed ii) different lithotypes showed different colonization rates; iii) No significant signs of colonization were observed on the surface of Oira stone; iv) microbial cells occurred in densely packed EPS-glycoconjugates aggregates surrounded by mineral particles; v) biofilms retrieved on different litiotypes are metabolically active.

Evaluation of the state of conservation of stone substrates in different biocolonization conditions

The stone elements of the top levels of the façade and of the spires are characterized by complex geometries, variable inclination of the surfaces and different exposure conditions with respect to orientation and susceptibility to direct rainfall. Due to their specific location in the façade, these elements are also the most intensively subjected to the action of environmental damaging mechanisms, in particular erosion by meteoric waters and wind and disaggregation by thermal expansion of grains induced by solar radiation and large thermal excursions. The overall state of conservation of the stone elements is therefore highly non-homogeneous, especially as far as the extent and coverage of microbial growth is concerned.

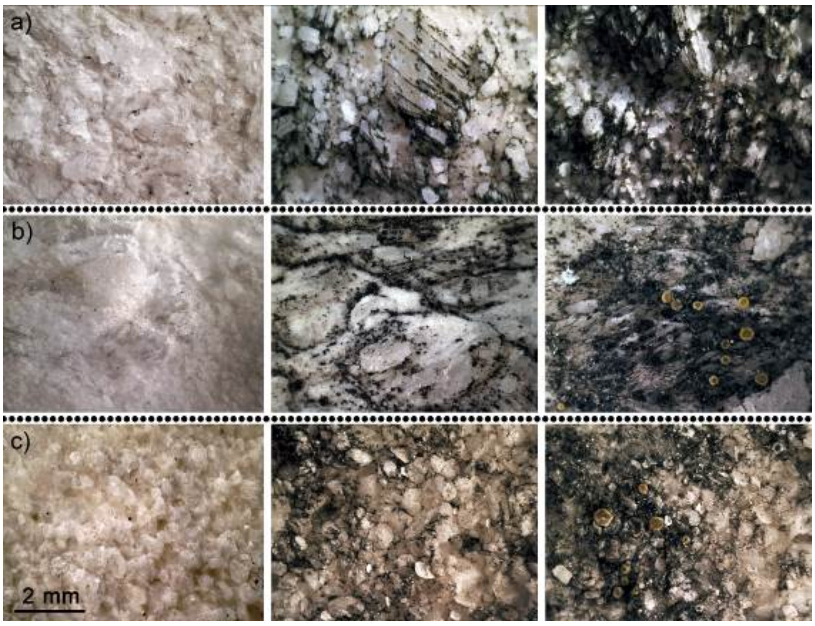


Fig. 5 - *In situ* microscopy documentation of the exposed surfaces of Candoglia (a), Musso (b) and Crevola marble (c) with no visible alteration (left column), and in presence of medium (central column) and intense (right column) biocolonization.

The *in situ* microscopic observation of the different marbles provides preliminary indications of the specific morphological features of not colonized surfaces with respect to adjacent ones subjected to medium and intense biocolonization (Fig. 5~~Fig. 5~~). Candoglia marble shows an irregular surface with enhanced roughness and a very limited surface deposition of dark particulate matter. The erosive effect of atmospheric exposure and rain-wash is particularly affecting the stone grains, which appear heavily detached and corroded. The presence of partly or almost completely detached quartz-like inclusion, which are usually strongly embedded in the crystalline structure of unaltered stone, is a further indication of the erosion process affecting the exposed front. The biological colonization exploits the presence of voids and discontinuities of the stone substrate as preferential location of growth. A dark-green biofilm is particularly concentrated along the grain boundaries and within the corroded grain surfaces, which are emphasized and can be more easily detected. A non-continuous and network-like pattern of biological growth (Gorbushina, 2007) can be observed as a result. In presence of intense biocolonization, the morphological features of the stone matrix become hardly detectable due to the formation of a thick biofilm.

The state of conservation of the non-colonized Musso surfaces is characterized by deterioration patterns similar to those observed for Candoglia. Advanced grain disaggregation and corrosion can be detected at the expenses of the grain boundaries and exposed grain surfaces, leading to an increased roughness but less intense than Candoglia one. The biocolonization appears as dark formations of spot-like agglomerates particularly located along micro-cracks and grain borders. Areas with the highest concentration of colonization show a more homogeneous biofilm growth overlapped to the substrate. Several isolated yellow spots due to lichen proliferation are also formed over the biofilm.

The atmospheric deterioration of Crevola marble is associated to the progressive loss of adhesion and detachment of crystalline grains from the medium to fine-grained exposed matrix. Erosion with loss of material resulting from superficial granular disaggregation is the main deterioration patterns observed on Crevola. The colonization in this case does not preferentially develop within the grain boundaries or irregularities of the substrate, but it tends to cover the surface. A dark-green to black spot-like biofilm is associated, as in the case of Musso marble, to yellow isolated lichens of sub-millimetric dimension.

Due to its quite different compositional nature with respect to the marbles, Oira stone is, as expected, much more durable under the same outdoor exposure conditions. Its state of conservation is mainly affected by the action of meteoric water. The specific deterioration mechanism of Oira stone has been studied in a previous work (Gulotta et al., 2017) and it involves the selective leaching of magnesium from the phyllosilicate structure of lizardite. As a result, a distinctive colour alteration of the most external material occurs as well as limited scaling of the surface, but neither loss of mechanical cohesion of the outermost stone layer nor traces of biological colonization are observed.

The differential state of conservation of non-colonized and biocolonized samples has been studied by SEM-EDX analysis, both on the external surface and on polished cross-sections. Two main aspects have been specifically investigated: i) the extent and type of damage of the crystalline structures; ii) the type of interaction of the biofilms with the stone substrate with respect to the prevailing location of growth (epilithic and/or endolithic development).

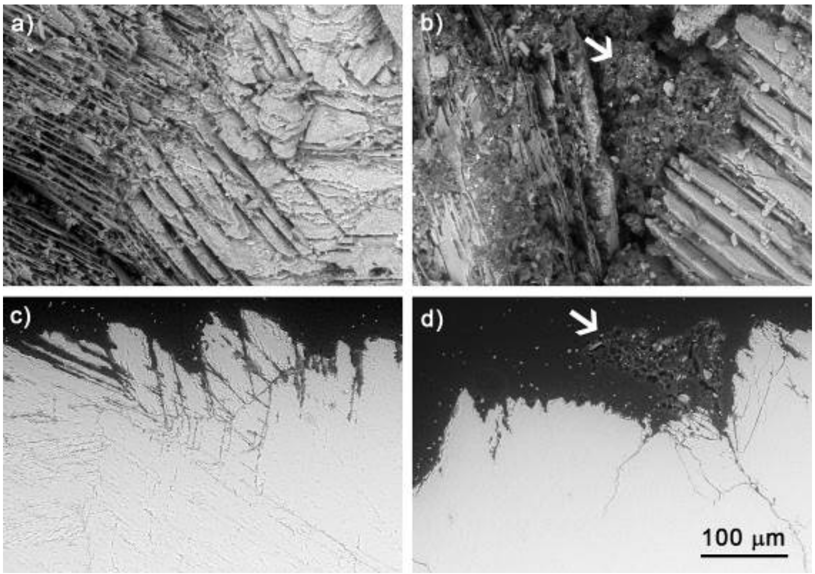
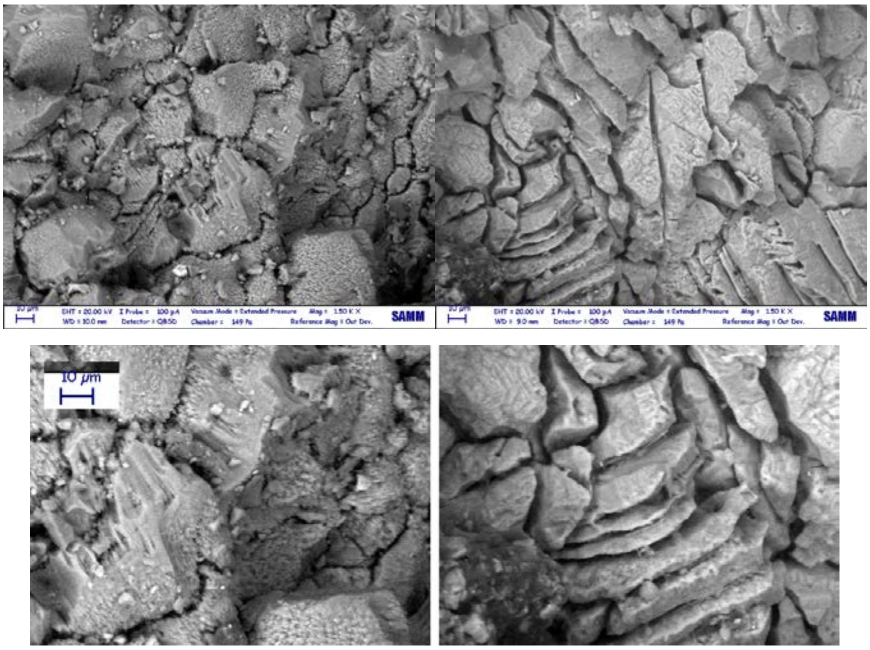
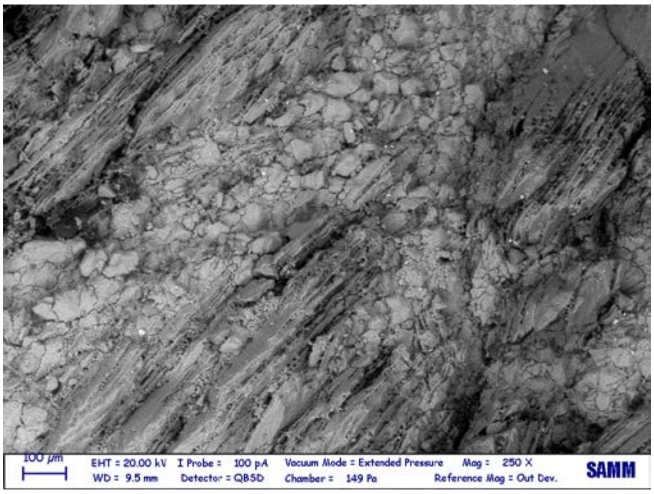


Fig. 6 - SEM images of the microstructural features of Candoglia marble: exposed surface of stone fragments without (a) and in presence (b) of biocolonization; polished cross-sections without (c) and in presence (d) of biocolonization Arrows indicate clusters composed of biological structures and soil dust

The non-colonized Candoglia marble (Fig. 6~~Fig. 6~~a,c) shows a highly corroded crystalline matrix, with particularly irregular and rough grain surfaces crossed by a number of sub-parallel fissures. These correspond to preferential erosion and progressive detachment along the cleavage planes of the stone, according to a typical deterioration pattern affecting compact marbles exposed outdoor (Weber et al., 2007). Such weathering effects are mostly related to the combined long-term action of the rain runoff and of environmental thermal stress cycles (Siegesmund and Snethlage, 2011, Luque et al., 2011). The stone observed in polished cross-section shows highly cracked superficial grains and the presence of fissures down to a 200 µm depth. The inner bulk material still demonstrates a good residual cohesion and appears rather compact. The biocolonization is preferentially distributed along the widened grain boundaries and it fills the discontinuities and detachments along the cleavage planes (Fig. 6~~Fig. 6~~b,d). Biocolonization forms irregular agglomerates that embed atmospheric aerosols, that is carbonaceous, heavy metal and mineral particles rich in silicon, magnesium and aluminium. As far as the surface roughness at the microscopic scale of the grains surface is concerned, the colonized substrate shows a lower extent of corrosion. This is further confirmed by the polished cross-section observation, which shows a reduced amount of cracked grains and fissures than those observed in the exposed non-colonized sample. This is in accordance with findings by Hoppert and colleagues (Hoppert et al., 2004), which reported no evidence of increased weathering of carbonatic rocks after some years from biocolonization. The researchers proposed that continuous mineral dissolution would deprive the endolithic biofilm of protection and therefore would force the biofilm to penetrate deeper into the substratum. Another paper by Hoppert and colleagues (Hoppert and Konig, 2006) claimed that microbial pioneers actively dissolve minerals of pre-existing openings at the surface and small fissures. However, at a later stage of growth colonisers do not cause deterioration as colonization is successful when the surface is stable for years.





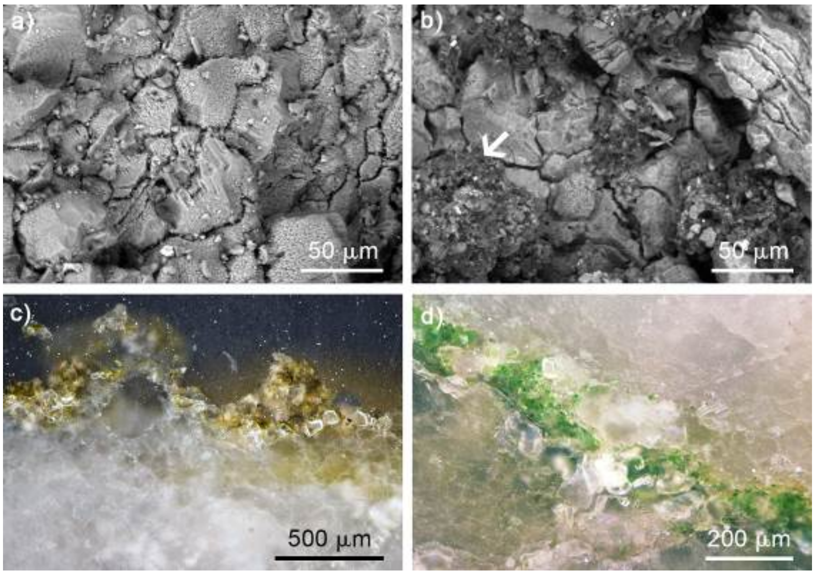


Fig. 7 - SEM images of the microstructural features of Musso marble: exposed surface of stone fragments without (a) and in presence (b) of biocolonization. Polished cross-section images of Musso showing superficial colonization (c) and endolithic growth within a micro-crack of the stone matrix (d)

The atmospheric deterioration of Musso marble induced an intense level of damage. The characteristic medium to coarse-grained and heterogeneous microstructure of the stone shows different type of prevailing deterioration patterns depending on the grain size: crystalline grains belonging to the smaller fraction mainly suffers from widening of the intergranular spaces and cracking, whereas the coarser ones show heavily corroded surfaces and detachments along the cleavage planes~~ (Fig. 7a)~~. Similarly to what observed on Candoglia, the extent of the corrosion of the grains surface is particularly intense on non-colonized substrates (Fig. 7~~Fig. 7~~a) and, on the other hand, it appears less advanced on the biocolonized ones (Fig. 7~~Fig. 7~~b). The biological growth forms isolated clusters enriched with mineral fragments, carbonaceous and metal particles. Polished cross-section observation reveals two deterioration patterns to be ascribed to biological growth (Fig. 7~~Fig. 7~~c-d): a superficial dark-brown biofilm with irregular thickness that follows the grains morphology; and an endolithic growth made of green coloured spherical aggregates developed in the bulk, which exploits the presence of some micro-cracks and grains decohesion of the crystalline microstructure. The extent of the granular decohesion is remarkable in both stone conditions (with and without biological colonization), as it reaches an average depth of about 2 mm.

The morphology of the deteriorated surfaces of Crevola marble is characterized by the progressive corrosion and detachment of the dolomite grains from the crystalline matrix. As a result, the grain boundaries are deepened and widened and the large flat phlogopite crystals originally packed within the crystalline matrix become exposed. Moreover, several trans-granular fissures cross the grain surfaces (Fig. 8~~Fig. 8~~a). The morphology of the colonized material does not show significant differences with respect to the previous condition. Grain boundaries, micro-cracks and voids are partially concealed by the biological growth (Fig. 8~~Fig. 8~~b), which is particularly rich in mineral and iron-rich particles (visible as rounded and bright spots in scanning electron microscopy). Surface dark-brown biofilm and endolithic intense green growths can be detected in cross-section observation. The diffused endolithic growth, in this case, is developed along the boundaries of the detached grains (Fig. 8~~Fig. 8~~c). The disaggregated stone matrix provides a network of interconnected micro-cracks down to 1 mm depth in the bulk, which are extensively colonized by biological material (Fig. 8~~Fig. 8~~d).

Commentato [UdMO1]: La differenza nel degrado tra a) e b) non è evidente sembrano uguali, stesso tipo di fratture e stessa grandezza dei grani distaccati. Queste immagini non sono convincenti.

Commentato [UdMO2]: La differenza nel degrado tra a) e b) non è evidente sembrano uguali, stesso tipo di fratture e stessa grandezza dei grani distaccati. Queste immagini non sono convincenti.

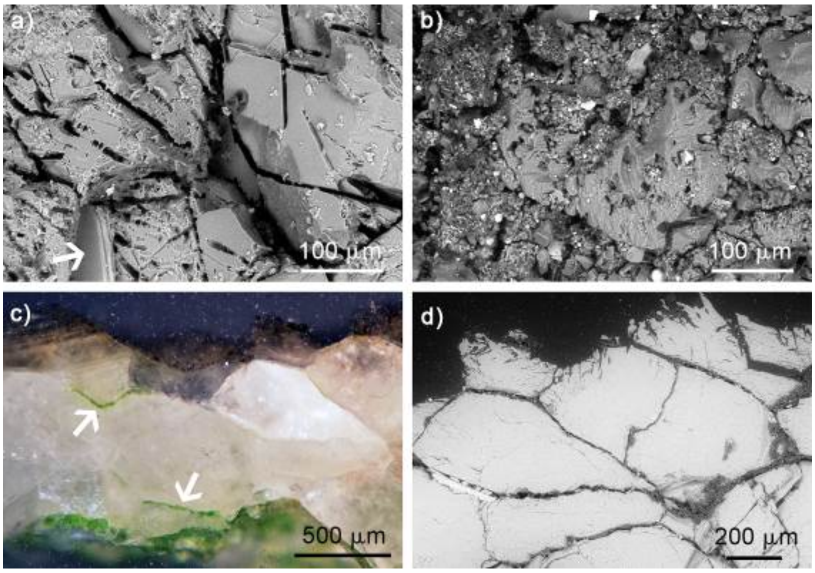


Fig. 8 - SEM images of the microstructural features of Crevola marble: exposed surface of stone fragments without (a, arrow indicates a flogopite crystal) and in presence (b) of biocolonization. Polished cross-section images in optical (c, arrows indicate endolithic biodeterioration) and electron microscopy (d) of Musso showing superficial colonization and extensive endolithic growth within a diffused network of micro-cracks in the stone matrix

Samples of biofilms formed over the stones have been analysed in order to highlight variations related to the different mineral substrates. FTIR analysis can provide insights into the overall compositional features of the biofilm but the presence of numerous cellular compounds results in overlapping and broadening of the absorption signals in the mid-IR range (Schmitt and Flemming, 1998, Yu and Irudayaraj, 2005). In Fig. 9~~Fig. 9~~ the FTIR characterization of biofilm fragments in the 1800-800 cm^{-1} spectral field is reported. According to (Schmitt and Flemming, 1998) two regions can be identified within this field, which are informative of the proteinaceous (1745-1450 cm^{-1}) and polysaccharide content (1085-1050 cm^{-1}) of the biofilm. Results indicate that a similar overall composition can be observed for all biofilms, which is in accordance with literature data (Yu and Irudayaraj, 2005, Chen et al., 2013, Tugarova et al., 2017). Peaks centred at 1653, 1546 and 1449 cm^{-1} are assigned to amide I and amide II absorptions of proteins and C-H bending mode of proteins and lipids. Peak at 1408 cm^{-1} can be attributed to the C-O-H bending mode of proteins and carbohydrates. The split band at 1073-1039 cm^{-1} is originated from the C-O and C-C stretching mode, C-O-C and C-O-H deformation of polysaccharides of the biofilms.

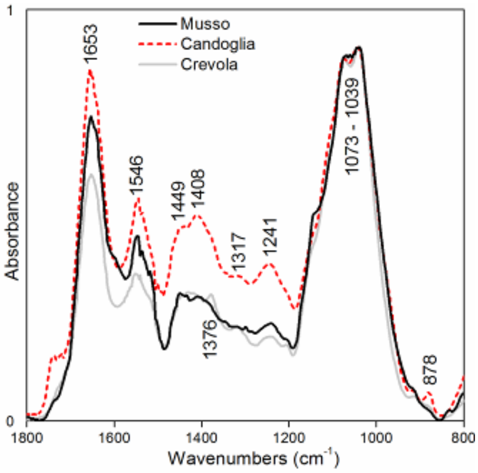


Fig. 9 - FTIR characterization of epilithic biofilms on carbonatic stones

Overall, the comparative evaluation of the state of conservation indicates that no major differences in the deterioration patterns and damage extent in each stone type can be observed as a result of biocolonization. Moreover, in case of Candoglia and Musso the most intense surface corrosion effects are associated to the non-colonized samples. Thus, biofilm formation and endolithic growth in the present condition cannot be considered as active and primary damaging mechanisms, although they can be held responsible for extensive colour alteration of the stone surfaces in case of Candoglia, Musso and, to a lesser extent, Crevola marble. Environmental exposure, in particular chemical and mechanical erosion due to precipitations, thermal stress and chemical alteration in polluted atmospheric conditions is the main driver controlling the damage. As in this work, Cutler and co-workers (Cutler et al., 2013) found that whilst the growth of green algal biofilms may have an aesthetic effect, they had little or no impact on the physical integrity of sandstone heritage structures.

Differential stone biosusceptibility and prevailing location of the biological growth can be correlated to specific stone features. Mineralogy of the substrates seems to play a relevant role in colonization (Miller et al., 2012), which is diffused to all carbonatic stones whereas the silicatic Oira stone shows the lowest bioreceptivity as indicated by microscopy observations and total biomass data (Fig. 3~~Fig. 3~~). This is in accordance with previous studies indicating that the presence of high amount of carbonate compounds in the substrate produces favourable conditions for microorganisms proliferation during primary biocolonization (Miller et al., 2006).

The rather different microstructural features of the weathered substrates, in terms of surface roughness, widening of grain boundaries and associated granular disaggregation of the stone matrix clearly influence bioreceptivity as well. The highest rate of epilithic growth on Candoglia marble (Fig. 3~~Fig. 3~~) is associated to

Commentato [UdMO3]: Dagli spettri FTIR mi sembra abbastanza evidente che la parte “proteica” del biofilm è sviluppata di più su Candoglia>Musso>Crevola in accordo con il trend visto già, a parità di substrato “energetico” polisaccaridico.
Questo mi pare importante dirlo.
Quindi si conferma: maggiore sviluppo di microorganismi su Candoglia>Musso>Crevola

Commentato [UdMO4]: Al contrario, in qualche modo sembra che la crescita del biofilm protegga la superficie e prevenga la perdita di materiale.

a particularly irregular surface, where extensive chemical and mechanical erosion has created preferential niches for microbial settlement and proliferation. As far as epilithic colonization is concerned, similar considerations can be drawn for Musso marble. In both stones, despite the long-term corrosion phenomena, the surfaces are not affected by significant loss of material and therefore they provide a rather mechanically stable environment for the biofilm. On the contrary, the reduced epilithic biomass on Crevola can be correlated to the extent of superficial granular disaggregation. Most of the biological growth therefore penetrates within the diffused network of micro-cracks exploiting primary chemical-physical weathering, and endolithic colonization prevails.

4. Conclusions

- A relationship between bioreceptivity, stone mineralogy and chemical-physical weathering can be observed. Silicatic stone are not affected by colonization whereas among the carbonatic ones, the calcitic Candoglia and Musso marble show the highest colonization rate. Increased superficial roughness and mechanically stable surfaces allow epilithic growth. Endolithic proliferation only occurs in partly or heavily disaggregated substrates and therefore exploits the effect of primary deterioration.
- Chemical-physical mechanisms are largely responsible for the stone damage, even in presence of extensive biological growth. The damage extent observed does not follow the colonization rate.
- (UNIMI) Rispetto a quanto detto in intro, la decisione su trattamento biocida non può essere esclusa, ma va valutata prevalentemente rispetto all'alterazione cromatica ed alla potenziale efficacia.

Commentato [UdMO5]: Questo ci vuole. Ok

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