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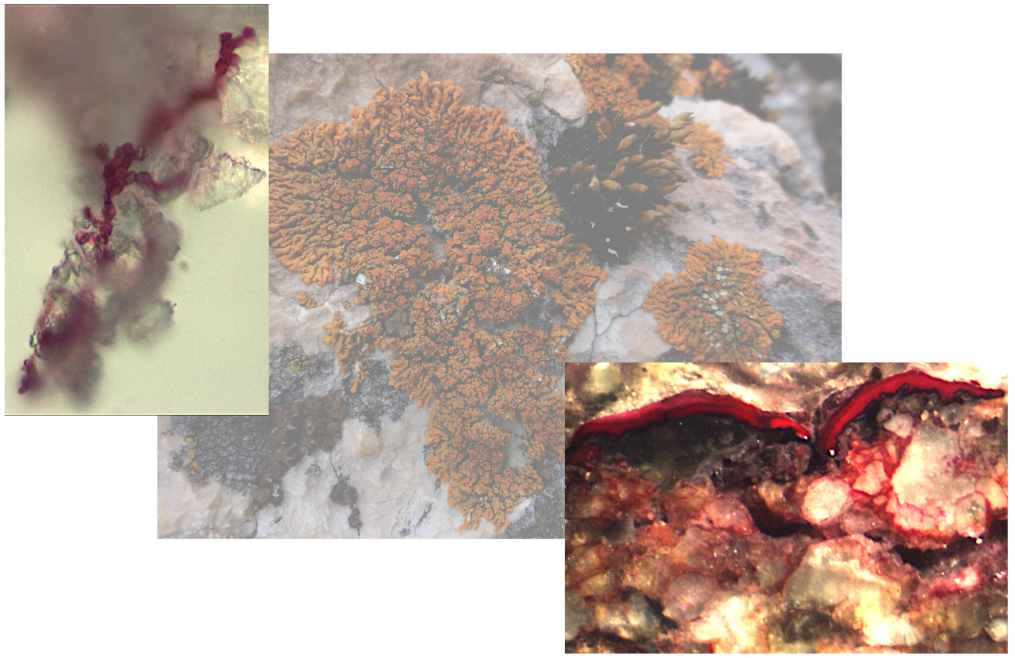

Lithobiont biodiversity, biodeterioration and growth control on cultural heritage

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33° Cycle: 2017 – 2021

Università degli Studi di Torino

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Preliminary remarks

Science and Cultural Heritage

The field of scientific research applied to cultural heritage materials is vast and inter-disciplinary and requires contributions from many different humanistic and scientific areas. This cooperation allows to obtain information with different perspectives, which merges together and completes each other, in order to return an integrated data interpretation. The role of scientists is particularly relevant in two areas of cultural heritage studies: the archaeometry and the conservation science. The conservation science deals with the study of the cultural heritage materials, with purposes of characterizing the deterioration processes affecting it in the past, in the present or that may take place in the future, and to suggest appropriate restoration procedures, suitable conservation treatments or preventive preservation approaches, respectively. The archaeometry can be defined as the field of study where scientific diagnostic analyses from the field of chemistry, physics, biology, earth sciences and engineering are applied to the knowledge of the material of cultural heritage, supporting the archaeological questions.

Such a rigid separation among conservation science and archaeometry is not always shared by experts in the field, and in some cases the conservation science is considered as a branch of archaeometry. These blurred boundaries are due to the intrinsic inter-disciplinary nature of the two fields and to the employment of similar techniques. In fact, when applied to cultural heritage, scientific methods and technologies have to undergo the need for analyses as non-invasive as possible on the original material. For this reason, the number of possible techniques are considerably reduced, and *ad hoc* non destructive or micro-destructive procedures have been developed specifically for the archaeometric and conservation study areas.

Next to the other scientific areas, plant biology offers useful knowledge and tools to contribute to conservation of cultural heritage. Biological

expertise allows to increase the knowledge of cultural heritage materials, such as wood, fibres, charcoal, paper, etc, which can be informative of source, treatments and age of the artifacts. Archaeobotany studies plant and microbial remains from archaeological sites, providing paleoenvironmental information to support and enrich archaeological interpretations. More remarkably -as at the focus of this PhD thesis- the materials of historical-artistic interest can be subject to biological colonization that can modify and weaken them. In indoor settings, including certain museum spaces, materials of biological origin may be exposed to microbial colonization that could easily biodeteriorate them, but also inorganic artifacts – made out of ceramic material, glass, stone, metal – can withstand to similar processes. In outdoor environment the biodeterioration issue is amplified and complex, ranging from micro- to macro- dimensions: surfaces can be colonized by microbial biofilms (as cyanobacteria, non lichenized fungi, micro-algae) and cryptogams (as lichenized fungi and mosses), but in wide areas, as archaeological sites, the roots of higher plants can also critically damage architectural structures, representing a threat to their solidity. The extent of biodeterioration caused by different (micro-)organisms depends on their morphological, physiological and ecological traits, but also on the nature of the affected material and the environmental conditions. The investigation of these issues with a thorough biological approach allows to better understand them and to study the most suitable conservative and preventive strategies.

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Introduction

1.1. Biology for archaeometry

Plant biology remarkably contributes to the study of materials of interest for cultural heritage, both in terms of identification of biological materials and their composition, and for the analysis of biodiversity in archaeological contexts. In the first case, materials identification can support provenance studies, datings, contextualisations, finishing treatments carried on the materials (see Paragraph 1.1.1). In the second case, the remains of ancient plant parts (archaeobotany) and microorganisms (paleomicrobiology), together with still alive microbial communities found in archaeological soils (archaeomicrobiology), can enrich the archaeological framework during excavations and data interpretation (see Paragraph 1.1.2).

1.1.1. Plant materials in cultural heritage

The majority of the organic materials of archaeological or historical-cultural interest has a plant origin. Their identification is mostly based on the morphological and anatomical features, thus needing a biological expertises (Caneva, 2005).

Archaeological wood can be found as dry, charred, waterlogged, fossilized and sub-fossil, depending on the excavation context. In each case, wood micro- and macro- structure is preserved, which allows to identify the material and to gain information on technological advances and commercial activities (Lev-Yadun, 2008). In particular,

dendrochronology makes possible to establish the age of wood artifacts with a very high precision by analysing tree-rings (Haneca *et al*., 2009).

Textile materials can also be found in archaeological context and are often derived from plant parts, such as linen, hemp and cotton. These materials are usually very fragile and their analysis is complicated by the deterioration and discoloration (Cybulska and Maik, 2007). However, it is often possible to determine their past appearance and to identify the original plant fibre, which is also useful for both material study and conservation purposes (Caneva, 2005). Paper is a further material derived from plants, which, along the centuries, has been manufactured starting from different plant parts, depending on the historical period and the geographical area (Caneva, 2005; Missori, 2016).

1.1.2. Archaeobotany and archaeomicrobiology

Archaeobotany studies aim to support archaeological interpretations by examining plant remains from excavations and recognizing the relationship between man and plants in the past (Lityńska-Zając, 2018). This research field also includes fallouts in the environmental sciences (environmental archaeology).

Finding plant materials in the archaeological layers allows a deeper comprehension of plant distribution and environmental change, plant domestication, past foodways, economical exchanges between populations (Fuller, 2020), and may offer information for dating the layers. The archaeobotanical materials can be macroremains, such as fruits, seeds and wood, or microremains, such as pollen, spores, diatoms, phytoliths and starch grains (Lityńska-Zając, 2018). The interpretation of these materials is not only related to the identification, but also to the

quantification, to assess the roles for the different plants.

The conservation of plant and other organic materials in archaeological soils is only allowed under peculiar depositional and burial conditions (e.g. soil waterlogging), while most of residues, with the exception of mineral(-ized) or charred components, is generally lost because of transformation by soil microorganisms (e.g. in well drained soils) (Jacomet, 2007; Xu *et al*., 2017). On the other hand, the soil microbiota may bear the record of the past environmental conditions and the activities of ancient people (Demkina *et al*., 2010; Peters *et al*., 2014, with refs. therein; Siles *et al*., 2018). In particular, microbiological investigations on archaeological soils have recently highlighted that bacterial and fungal community structures from anthropogenic layers are differentiated depending on the past human activities and may address their recognition and interpretation (archaeomicrobiology).

1.2. Biology for conservation

Organisms interact with materials, biodegrading and altering them. In the field of cultural heritage, these processes fall into the phenomenon of "biodeterioration", which is a term first proposed by Hueck (1965, 1968) with a negative sense as opposed to the neutral/positive sense of "biodegradation". "Any undesirable change in the properties of a material caused by the vital activities of organisms" is considered a biodeterioration effect and can be caused by animals, plants and microorganisms. Organic and inorganic materials can all undergo biodeterioration processes on a macro- and a micro- scale, both in indoor and outdoor environments. The biodeterioration of inorganic materials mainly occurs in outdoor environment, involving the materials used to realise architectural structures, covers, and sculptures. Wood, stone, metals and glass can be colonised by (photo)autotrophic and/or heterotrophic organisms, with consequences falling both on the aesthetic and the conservation of the artifacts.

Biodeterioration of stone in outdoor environment can occur on natural (rocks and minerals) and artificial (deriving from natural materials transformation, such as mortars, plasters, bricks, cements, etc.) materials and is caused by the growth of lithobionts (i.e. rock-dwelling organisms) on their surfaces and within their interior (Caneva *et al*., 2008). This interaction between stone and organisms can be expressed in many different ways, depending on the complex relationship among the lithobionts, the material and the environment.

In this PhD thesis different perspective of biodeterioration are analysed, focusing on different groups of lithobionts (described in Paragraph 1.2.1), the role of bioreceptivity and external factors on the

variability of lithobiontic communities with potential of biodeterioration (Paragraph 1.2.2), and the mechanisms of physico-chemical interaction between lithobionts and stone substrate (Paragraph 1.2.3). The influence of colonization, however, is not always negative. In some cases, it can be considered an aesthetic enrichment or it can bring an additional value in terms of biodiversity. Moreover, in some peculiar circumstances, lithobionts can exert bioprotective effects on stone surfaces. For these reasons, the balance between the different deteriorative and protective effects caused by lithobiontic colonization on stone (Paragraph 1.2.4) should always be evaluated, as key information to address decisions on their removal. When a removal intervention is necessary, methods to assess the most suitable control strategies (Paragraph 1.2.5) should also be considered.

1.2.1. Lithobionts involved in biodeterioration processes

While organic materials are often biodegraded by organisms exploiting them as a nutritional source, lithobionts usually use stone surface as a support for their growth. For this reason, the majority of lithobionts in outdoor environment are autotrophic organisms, such as algae (Ortega-Calvo *et al*., 1991), cyanobacteria (Crispim and Gaylarde, 2005; Albertano, 2012), lichens (Seaward, 2015; Salvadori and Casanova-Municchia, 2016), bryophytes (Ricci and Altieri, 2008) and vascular plants (Pawlik *et al*., 2016). However, if there is an external supply of nutritional substances, some heterotrophic organisms, such as non-lichenized fungi, can be found on stone surfaces (Sterflinger, 2010), and in some cases they even represent a major threat for conservation. Lithobionts challenge very stressful situations on stone surfaces, which are extreme

environments in some cases subjected to sudden changes. They have been defined as “poikilo-tolerant” organisms to highlight their ability to resist to variable stresses (Gorbushina, 2007).

Different groups or even different species from the same group can promote different biogeophysical and biogeochemical processes which can produce different biodeterioration impacts. Moreover, microorganisms are often associated in heterogeneous **biofilms**, embedded in a matrix of extracellular substances which mediates the interaction between the organisms and has other functions. Biofilms can be dominated by different groups of organisms, affecting biofilm properties and its interaction with substrates. The identification of the lithobionts with molecular, microscopy and spectroscopy methods (Sanmartín *et al*., 2018) allows a correct interpretation of the on-going processes.

In the framework of macro- and micro-organisms inhabiting stone surfaces and contributing to the biodiversity of monumental and archaeological sites, some major or marginal focus of this PhD thesis is dedicated to the following groups.

Algae and **cyanobacteria** are both unicellular photosynthetic microorganisms that can commonly be found above stone surfaces, where they establish as first colonizing communities (pioneer colonists) (Pinna, 2017). While algae are usually green, due to the presence of chlorophyll *a*, cyanobacteria produce a variety of pigments. Their characteristic blue-green colour is due to photosynthetic pigment composition of chlorophyll *a* (green), carotenoids (yellow-orange), phycocyanin (blue) and allophycocyanin (blue-green), but they can also be from red to pink, due to the presence of phycoerythrin pigment (Vázquez-Nion *et al*., 2013). Since they do not need organic substances,

these autotrophic organisms easily develop on oligotrophic stone surfaces, only limited in their growth by light and water availability (Macedo *et al*., 2009), even if the quality of solar irradiation, the adequate temperature and the atmospheric deposition can also be relevant (Tomaselli *et al*., 2000). For example, low-light environments, such as caves, display a much lower biodiversity, characterized preferentially by fast-growing eukaryotic algae (Mulec, 2005; Mulec and Kosi, 2009). To avoid desiccation, algae and cyanobacteria grow in colonies, often associated, producing an extracellular matrix of proteins and sugar polymers (EPS) which embed the cells, cementing them together (Pinheiro *et al*., 2019). EPS matrix also has roles for adherence of colonies to the substrate, protection from UV radiation and nutritional source and protecting them from desiccation (Rossi and De Philippis, 2015).

In particularly harsh conditions, cyanobacteria can also develop with an endolithic colonization, finding protection from environmental stresses below the stone surface (de los Ríos *et al*., 2003; Wierzchos *et al*., 2006).

Lithobiontic fungal component is represented by two major groups of **fungi**. Fungal communities dominated by hyphomycetes are mostly present in moderate-humid climates, while microcolonial fungi (MCFs) dominate the fungal community in arid and semi-arid conditions (Sterflinger, 2010), such as monument surfaces in temperate climates. These latter microorganisms are a polyphyletic group of Ascomycetes which share morphological plasticity and functional adaptive traits to withstand extreme conditions such as high UV radiation, scarcity of water and nutrients, high humidity, temperature variability and oxidative stresses (Isola *et al*., 2016). Their resistance mechanisms are related to

their particular growth forms, their meristematic growth and the production of melanin (de Hoog, 1993). MCFs are characterized by swollen, "torulose" hyphae (De Leo *et al*., 2019) which minimise the contact with the atmosphere, thus limiting dehydration, and develop by isodiametric enlargements of cells and subsequent division (meristematic growth), with thick melanin deposits in the walls that protects the cells during the germination phases (Wollenzien *et al*., 1995, 1997). Melanin production has been related to photoprotection, resistance to oxidation, thermoprotection, energy harvesting and metal binding roles (Gorbushina and Broughton, 2009; Pacelli *et al*., 2017). MCF stress tolerance ability allows their survival in harsh conditions including many habitats in urban, polluted environment, such as monuments, statues, roofs, building façades and even photovoltaic panels (Marvasi *et al*., 2012; Shirakawa *et al*., 2015; Ruibal *et al*., 2018), and also impacts on their resistance to biocides (Gorbushina *et al*., 2003).

Lichens are a self-sustaining partnership between a fungus (mycobiont), eukariotic micro-algae and/or cyanobacteria (photobiont) and associated bacteria and parasitic or saprotrophic fungi (Grube *et al*., 2015). The mycobiont, more abundant, adheres to the substrate and protects the photobiont, while the photobiont produces the nutrients for the entire lichen (St. Clair and Seaward, 2004). Lichen thallus has a stratified structure, characterized by a superficial layer of dense mycobiont hyphae (cortex) that protects the photobiont layer, followed by a less dense layer of hyphae (medulla). In crustose lichens, which are the most diffuse lichen growth form on stone substrates, hyphae from the medulla layer directly penetrate into the substrate (Favero-Longo *et al*., 2005).

Lichens can reproduce sexually, by ascospore dispersion, and by vegetative dispersal, which can happen by means of specific structures (soredia, blastidia, isidia) or via thallus fragmentation (Nash, 2008).

Lithobiontic lichens are characterized by a very slow growth rate and a high stress tolerance. This latter characteristic allows them to survive in very harsh environments, with high UV radiation, drought and thermal excursion, such as hot and cold deserts, polluted urban environments and even space (Gilbert, 2000; Sancho *et al*., 2007; Pinna, 2017). The high extremotolerance is related to adaptive mechanisms which allow them to survive desiccation by shifting into anhydrobiosis state (Kranner *et al*., 2005) and also enhance UV and high/low temperature tolerance (Nybakken *et al*., 2004). Their morphological and anatomical traits have also been suggested as involved in the extremotolerance mechanisms (Meeßen *et al*., 2013), and the production of secondary metabolites is another factor influencing their tolerance to stresses (e.g. Solhaug and Gauslaa, 2004; McEvoy *et al*., 2006). Secondary metabolites specifically produced by lichens are more than 1000 (Stocker-Wörgötter, 2008), have a low molecular weight and are accumulated in the cortex or in the medulla layer. The functions of these substances are very heterogeneous (cations reserve, detoxification, anti-herbivores function, structural support, UV protection, etc.; Elix and Stocker-Wörgötter, 2008; Yamamoto *et al*., 2015) and have not always been clarified. A very diffused lichen metabolite is oxalic acid, which in the past was often considered a waste metabolite, but it is instead involved in many functional roles, from detoxification to the cycling of metals and other elements, mineral dissolution and mineral formation (Gadd *et al*., 2014).

1.2.2. Intrinsic and extrinsic factors influencing colonization

When dealing with stone colonization and biodeterioration, there are three important factors to consider: *i.* the properties of the colonising lithobionts (Paragraph 1.2.1), *ii.* the stone bioreceptivity, and *iii.* the macro- and micro- environmental parameters (Sanmartín *et al*., 2021).

Stone surface represents a very harsh environment for all groups of lithobionts, even if they only exploit it as a growth support, but the properties of some lithic materials make them more prone to colonisation. The stone aptitude to be colonised by (micro-)organisms, even without actual biodeterioration consequences, was defined by Guillitte (1995) as "**bioreceptivity**", and depends on the totality of material properties involved in (micro-)organism establishment, anchorage and development.

Both petro-physical and -chemical properties are involved in stone bioreceptivity. The most important physical properties are related to water movement and availability (Miller *et al*., 2012). Pore size and pore connection influence capillarity from the ground, water absorption and its retention, which support microbial growth and the spread of microflora through the moisture (Warscheid and Braams, 2000). Moreover, the presence of intrinsic discontinuities (pores, but also cracks, grain boundaries, etc.) of dimension compatible with microbial structures has been related to an increased presence of biomass within the stone (Favero-Longo *et al*., 2009). These discontinuities may rise in quantity and dimensions due to (bio)weathering conditions, further increasing stone bioreceptivity (secondary bioreceptivity) (Urzì *et al*., 2000; Jim and Chen, 2011). Surface roughness too is related to water retention, but also facilitates the microbial anchorage (Prieto and Silva,

2005) and provides them sheltered micro-habitats (Cámara *et al*., 2014). Stone mineralogical composition is another relevant factor for bioreceptivity, exerting an influence on biodiversity (Favero-Longo *et al*., 2011; Olsson-Francis *et al*., 2012). Finally, the presence of nutrients, even deriving from compounds related to conservation treatments, increase stone bioreceptivity (tertiary bioreceptivity) (Jurado *et al*., 2014; Sanmartín *et al*., 2019).

Other factors influencing stone colonization, and thus biodeterioration, are **environmental parameters**, such as climatic conditions, surface orientation, sun and wind-driven rain exposition, nutrient and water availability, supply of biological propagules (Pinna, 2017; Favero-Longo and Viles, 2020). These parameters influence propagule dispersion, establishment of new colonies and their development (Giordani *et al*., 2014).

Regional climate conditions are known to affect biological communities, influencing the velocity of colonization, the community dynamism, the biodiversity, the biomass, the patterns of interaction with the materials and also increasing stone bioreceptivity (Caneva *et al*., 2008). However, in the same climate conditions many differences can be found, due to local factors. Sunny surfaces represent a very hostile environment, with temperatures ranging at least from -45°C to +60°C and subjected to intense UV and infrared radiation during sunny days (Gorbushina, 2007). In contrast, in shaded areas biomass is usually higher, due to more favourable conditions for life (Ramírez *et al*., 2010). Besides temperature excursion, the average temperature reached by stone surfaces also influences biodiversity (Cutler *et al*., 2013; McIlroy de la Rosa *et al*., 2013). Wind-driven rain, water run-off, damp rising and water stagnation have been widely proven to affect water availability

and retention, which has important fallout for colonization (e.g. Abuku *et al*., 2009; Giordani *et al*., 2014; Traversetti *et al*., 2018). Some parameters can cause both positive and negative effects on colonization: for example, high local water run-off increases the dispersion of propagules, but negatively influences colony establishment (Giordani *et al*., 2014). Propagule dispersion as bioaerosol is instead influenced by wind intensity and direction (Mandrioli *et al*., 2003), and can also reach semi-confined outdoor environments, depending on the exchanges with outdoor environment and air circulation, related to room dimension (Zhang and Chen, 2006; Roccardi *et al*., 2008). Biodiversity is also influenced by surface inclination, which affects water retention and microbial establishment, with horizontal surface more rich in number of species and biomass with respect to inclined and vertical ones (Kumbaric *et al*., 2012; Blanco, 2014).

1.2.3. Biodeterioration processes

The first and immediate reason for conservators to remove or control lithobiontic growth is related to aesthetic modification of the surface, which alters the perception of the artifact, for example obscuring carved details (Pinna, 2017). However, besides the discoloration effect or the obvious damage due to plant roots, microorganisms exert damage below stone surface, in a range that depends on the lithobiont, the bioreceptivity and the environmental factors, and that represents the actual interface between biotic component and mineral material (Favero-Longo and Viles, 2020). At this interface, biogeochemical and biogeophysical processes take place, leading to stone disaggregation and alteration which is vital to pedogenesis (Jones, 1988) and landscape

shaping (biogeomorphology; Viles, 2020), but unwelcome on cultural heritage surfaces. The following sections include general insights on the main biodeterioration processes driven by photoautotrophs and fungi, which are mostly considered in the research lines of this thesis. Chemolithotrophic bacteria, which have been early considered -since 1950s- as stone biodeteriogens, are not treated in this thesis.

Biogeophysical processes cause mechanical fracturing, substrate defoliation and mineral disaggregation (Favero-Longo and Viles, 2020). Plant root systems can grow directly on pavements and masonries, also penetrating down to hypogean structures, for example in archaeological sites, where roots pressure directly endangers structural stability (e.g. Caneva *et al*., 2009; Bartoli *et al*., 2017). The damage is exacerbated by biological structures contractions and expansion related to water retention and the acidic action of root apices (Caneva *et al*., 2008). Also moss adhesion structures have been related to mechanical damage of historical surfaces, which they penetrate with rhizoids (Ricci and Altieri, 2008). In crustose lichens, mycobiont hyphae penetrate stone substrate up to several millimeters (Favero-Longo *et al*., 2005; Sohrabi *et al*., 2017). The depth and the pattern of the penetration depends on lichen growth form, that can be epilithic, endolithic or intermediate. The endolithic habit can be chasmo-endolithic (growth exploiting internal cracks), crypto-endolithic (growth within intrinsic porosity) or eu-endolithic (active mineral dissolution). In each case, the biomass beneath the stone surface causes internal pressure, increased and decreased in correlation to water retention, causing defoliation (Caneva *et al*., 2008). The biogeophysical deterioration caused by algae, cyanobacteria and fungi is too related to cycles of drying and moistening (Sáiz-Jiménez *et al*., 1999). These microorganisms are often associated in biofilms, where

EPS drive the interactions with the substrate and increase the phenomenon of volume changes related to water (Negi and Sarethy, 2019). Moreover, algae and cyanobacteria can display endolithic growth (Gaylarde *et al*., 2017; Wierzchos *et al*., 2018), which in some cases is hidden by surface growth (Pentecost, 1992) or abiotic patinas (Pereira de Oliveira *et al*., 2008), and that can present major issue for its removal. Fungi, and in particular microcolonial fungi, can deeply penetrate stone surface, exploiting pre-existing discontinuities or actively perforating minerals (Sterflinger, 2000; Gadd, 2007). Finally, lichen growth has also been related to thermal stress, due to the colour of the thalli, different from the rock surface, which, due to sun irradiation, provokes local hotspots that can be correlated to mechanical disaggregation and weathering (Carter and Viles, 2004).

The chemical destabilization and mineral alteration due to **biogeochemical processes** of deterioration are related to primary and secondary metabolites secreted by organisms. All (micro-)organisms, as a consequence of carbonic anhydride release and reaction with water, produce carbonic acid, which creates an acidic environment and mobilize cations (Ortega-Morales *et al*., 2000; Crispim and Gaylarde, 2005; Ricci and Altieri, 2008). Biopitting (i.e. formation of round cavities of sub-millimetric dimensions) caused by endolithic lichens is usually due to the development of reproductive structures (peritecia) beneath stone surface, and in some cases carbonic acid-induced substrate acidification has been proved to be sufficient to provoke it (Weber *et al*., 2011). Mineral dissolution can be caused by other fungal primary metabolites, such as citric and malic acid (Wei *et al*., 2012; Sazanova *et al*., 2016). Another primary metabolite, typical of many lichens, is oxalic acid, an intermediate product of cellular metabolism. It causes phenomena of

biomineralization by combining with cations (Ca, Mg, Fe, Mn), to produce oxalates. Among them, calcium oxalate has a very low solubility and therefore can be found on many lithologies, even those poor in calcium. Calcium oxalate crystals precipitate in the inter-cellular spaces inside lichen thallus, at the lichen-stone interface or into the stone and can form biomineral patinas very persistent and difficult to remove (Caneva *et al*., 2008).

Lithobionts also produce a wide variety of secondary metabolites, including additional organic acids. Lichens, but also MCFs, secrete other chelating substances, such as siderophore-like compounds, involved in iron nutrition and also able to mobilize calcium, if under iron limitation (Favero-Longo *et al*., 2011). Such iron-chelating compounds might be responsible for pitting and etching on silicates and carbonates (Salvadori and Casanova-Municchia, 2016). Corrosive compounds contained in the EPS of MCF (Breitenbach *et al*., 2018) and cyanobacteria (Bellezza *et al*., 2006; Albertano, 2012) likely have roles in biodeterioration phenomena, which might be related to nutritional requirements.

1.2.4. Biodeterioration-bioprotection balance

The improvement of analytical methods in molecular biology has greatly increased in recent years the number of known lithobionts reported on and within stone materials (Sanmartín *et al*., 2018; Marvasi *et al*., 2019). However, microbial presence on lithic surfaces is not always related to a biodeterioration processes, since sensitive DNA-metabarcoding analyses also highlight microbial past traces, contaminants and microorganisms which do not interact with the material (Pinna, 2017; Favero-Longo and Viles, 2020). Besides these

"neutral" effects on stone stability and the previously described biodeterioration effect (Paragraph 1.2.3), bioprotective roles for biological patinas on stone surfaces have also been suggested, and, in the last two decades, increasingly deepened and understood.

Plants are mostly known for the biodeteriorative role of their root structures, but ivy has been proved to exert a potential bioprotective role on stone walls, for example absorbing atmospheric particulate (Sternberg *et al*., 2010) or buffering the damage due to thermal cycles and frost events (Coombes *et al*., 2018). An "**umbrella-like**" effect, i.e.the presence of lithobionts as a physical barrier that protects stone from abiotic weathering factors, has been identified for some plants (Sternberg *et al*., 2010; Coombes *et al*., 2018) and other (micro-)organisms. For example, lichen cover on limestone showed evidence of lowering solutional weathering (McIlroy de la Rosa *et al*., 2014).

Lichen colonization has also been related to **case-hardening** effects. Endolithic lichens in arid environments displayed iron oxides precipitation within stone discontinuities which, together with hyphae, filled the voids, cementing the material (Guglielmin *et al*., 2011). Similar cementation patterns were detected for epilithic lichens in more temperate and humid climate, precipitating a silica-rich layer in stone fractures and pores (Lee and Parsons, 1999). The varnish formation due to MCF melanins and EPS ability to bind metals, such as manganese, has also been documented and related to case-hardening (Gorbushina, 2003; Parchert *et al*., 2012). Heterogeneous biofilms too can harden stone surfaces, such as in the case of cementation caused by cryptoendolithic biofilms of fungi and cyanobacteria (Viles and Gourdie, 2004) or activity of bacteria and hyphomycetes, which cause the formation of rock

coatings in dry climate, also incorporating MCFs (Taylor-George *et al*., 1983).

However, in many cases the bioprotective effect is not clearly preponderant with respect to the biodeteriorative one. For example, endolithic lichens re-precipitate a layer of biogenic microcrystals of calcite above the algal layer for protection purposes (Pinna *et al*., 1998). This layer (*lithocortex*) has hydrophobic properties which can have bioprotection effects on the stone, but, when the lichen dies, these properties are lost and biopitting phenomena often occurs (Caneva *et al*., 2008). Moreover, *lithocortex* originates from the dissolution of the stone by the endolithic lichen, so a biodeteriogenic processes has to occur to allow the formation of the bioprotective layer.

Bioprotective effects of biogenic patinas of calcium oxalates are similarly discussed, because of the possible shielding effects from wind and water run-off (Souza-Egipsy *et al*., 2004; Gadd and Dyer, 2017), also related to the low solubility of these salts in water and even in acid rain (Biscontin *et al*., 1996). However, this potential effect comes at the expense of the surface from which they extract calcium ions (Rampazzi, 2019).

The equilibrium between calcite dissolution and calcite neoformation in fungi and lichens is discussed in many case-studies. In some cases they counterbalance each other (Bungartz *et al*., 2004), in other cases the equilibrium is on the side of bioprotection (McIlroy de la Rosa *et al*., 2014) or biodeterioration (Casanova-Municchia *et al*., 2018). This debate shows the importance of avoid general consideration of "biodeterioration or bioprotection", when dealing with stone colonization, since the two effects are not in contrast, but represent a balance among different aspects of (micro-)organisms-stone interaction

(Favero-Longo and Viles, 2020).

1.2.5. Growth control strategies

As the paragraph on the balance between biodeterioration and bioprotection processes (Paragraph 1.2.4) already hinted, the **necessity of a removal** of colonization from stone surfaces should not always be taken for granted. If the removal treatment is considered to preserve stone durability, scientific evidence should support the suspected negative impact of colonization, since lithobionts may display a neutral or even bioprotective effect on surfaces (Nugari and Salvadori, 2003). Moreover, in some cases the presence of lithobionts decreases stone durability, but their removal could further accelerate the biodeterioration process (Caneva *et al*., 2008). Beside the consideration on durability, a removal is more often considered for aesthetic reasons (Brimblecombe and Grossi, 2005). Even in this case, however, there are cultural aspects to take into consideration, related to the perception of stone cultural heritage (DeSilvey, 2017). In fact, in some contexts a surface colonization can be seen in a positive light, enhancing the merging of artifacts and nature and exalting their ancientness. A last reason to carefully consider potential removal is related to the additional values that lithobiontic communities can bring to biodiversity, in particular in archaeological sites (Nimis *et al*., 1992).

When an **intervention of growth control** is planned, it is possible to proceed in different ways. An indirect approach could aim to modify the (micro-)environmental parameters influencing the colonization. For example, in a cave with algal patinas caused by artificial illumination,

lamps with different emission spectra could limit microbial biomass (Albertano and Bruno, 2003; Sanmartín, 2021). Temporary or permanent shelters, mainly used in archaeological context, could reduce water availability and, depending on their transparency, shield sunlight and consequently discouraging colonization (Caneva *et al*., 2005; Doehne and Price, 2010; Salvadori and Charola, 2011). However, these methods should be carefully calibrated in shape and material, to avoid worsening the biodeterioration, as by introducing a greenhouse effect (Pinna, 2017). Interventions aimed to decrease stone bioreceptivity are also possible, for example treating the surfaces with water repellents (Pinna *et al*., 2012) or titanium oxide (Gladis and Schumann, 2011).

The described methods can be applied to eliminate the colonization, but are more often used for its prevention. To remove colonization, more direct methods are usually applied, which can be more invasive and thus should be carefully evaluated (Nugari and Salvadori, 2003). This calibration should consider (i) the nature of the microbial patinas, due to the different interaction patterns carried out by microorganisms (Paragraph 1.2.3); (ii) the colonized material, which could have different reactions to treatments (Young *et al*., 1995; May, 2010); (iii) the (micro-)environmental conditions and the structural geometry of the artifacts. For example, a biocide could be washed away by water run-off (Gromaire *et al*., 2015) or a heat-shock treatment would require external summer temperatures at middle-latitudes (Tretiach *et al*., 2012).

Removal of organisms and biological patinas from stone surfaces should be aimed to prevent a

(re-)colonization, but also to have a minimal impact on material integrity (Tretiach *et al*., 2007).The methods for the elimination of biological growth can be mechanical, physical or chemical. Mechanical

methods, such as cut and extirpation, can be effective on plants and mosses. On the contrary, mechanical methods carried on biofilms and lichens, such as brushing, scalpel cleaning, sand blasting and low-pressure washing (Pinna, 2017), may be ineffective on the microbial structures within the stone (Sohrabi *et al*., 2017), or even worsted the biodeterioration conditions, by spread the vegetative propagules (Seaward, 2015). For these reasons, these methods should be combined with other methods of devitalization, and used only to remove dead biomass after treatments (Pinna, 2017).

Physical devitalization methods can apply electromagnetic irradiation, heat and lasers and represent the evident advantage to devitalize the organisms with no impact on the environment and low risks on human health (Pinna, 2017; Cappitelli *et al*., 2020; Lo Schiavo *et al*., 2020). UV wavelenghts, largely used in food and health contexts, are mostly effective on surface colonization, with limited efficacy in depth (Berti *et al*., 2008; Kigawa *et al*., 2010). Monochromatic or moderate intensity lights have been used to kill fungi and cyanobacteria or to inhibit the growth of photosynthetic microorganisms (De Lucca *et al*., 2012; Hsieh *et al*., 2014; Sanmartín, 2021), but LED lights may dramatically change colour perception, for example in archaeological hypogea (Albertano *et al*., 2004). Laser ablation produces thermal, chemical and physical damage, is very selective and can be calibrated to penetrate to different depths in the stone (DeCruz *et al*., 2009; Speranza *et al*., 2013), but can modify and damage stone surfaces (Zafiropulos *et al*., 2003). Interesting results have been reached with heat shock treatments, exploiting the vulnerability to heat of poikilohydric organisms when hydrated, by leaving the patinas under the sun (Tretiach *et al*., 2012) or with microwave heating (Cuzman *et al*., 2013).

The use of chemical **biocides** derives from the healthcare and the food industry fields (Caneva *et al*., 1996), and belong to a large number of compound classes (Pinna, 2017; Lo Schiavo *et al*., 2020). Nowadays, the chemical devitalization methods are the most used and diffused, but also present many crucial issues, which influence the choice of the biocidal treatment. Ideally, biocides should be able to efficiently kill the target organisms, but without interfering with the stone, the operator and the environment, and maintaining a long-lasting effect (May, 2010; Cappitelli and Villa, 2021).

Accordingly, the lower possible interaction with stone material is of primary importance. The treated stone surface should not cause any chromatic alteration (Gazzano *et al*., 2013), and biocide and solvent should not interact with mineral structure and integrity (Caneva *et al*., 2008). Moreover, the residual presence on stone material of some biocides, such as quaternary ammonium salts, and polymeric consolidants (Cappitelli *et al*., 2004; Favero-Longo *et al*., 2018) could also be exploited as a food source by microorganisms, increasing stone bioreceptivity (quaternary bioreceptivity; *sensu* Sanmartín *et al*., 2021).

Different organisms may have different reactions to the same biocides. A compound toxic for a group of organisms might be harmless to another, and, furthermore, species-specific sensibility has also been confirmed in multiple studies (Tretiach *et al*., 2012; Stupar *et al*., 2014; Favero-Longo *et al*., 2017; Pfendler *et al*., 2018). Another critical point regarding toxicity is related to biocide amount, which should carefully be calibrated, to avoid overabundant quantities which would not increase the toxic effect on the target organisms, but could endanger the environment (Pinna, 2017).

Environmental conditions, related to regional climate and to micro-environmental factors, such as orientation and architectural surfaces

(Caneva *et al*., 2008), exert their effect mainly on biofilm and lichen hydration (Salvadori and Charola, 2011). The biocidal treatment on hydrated biofilms of cyanobacteria and algae, compared to the treatment on dry surfaces, was more effective (Charola *et al*., 2012), but pre-hydration has also been related to a faster wash-away of the biocide (Sterflinger and Sert, 2006). Lichens, as poikilohydric organisms, are stress tolerant when dry and sensitive when wet (Tretiach *et al*., 2012), suggesting that a biocidal treatment could be more efficient in hydrated conditions, although this point still needs to be experimentally verified (see Chapter 5). In the same way, the efficiency of biocide wash-off after the treatment, related to a more limited interference with the stone, is not clarified yet (Nugari and Salvadori, 2003; Pinna, 2017).

Lastly, stone structure, porosity and deterioration conditions influence the efficacy of biocidal treatments. These properties are related to stone ability to absorb the biocide, which is then more slowly released, exerting its effect on colonization for longer periods of time.

As a consequence, biocidal treatments should carefully be calibrated to bear their devitalizing effect on lithobionts. This issue is also particularly relevant in order to avoid the development of resistance phenomena by the lithobiontic communities. In fact, an inefficient use of biocides could select the most resistant species, which can potentially increase the biodeterioration-related issues and further complicate the conservation interventions (Pinna, 2017; Berman and Krysan, 2020). A laboratory phase of experimentation of biocide efficacy has been warmly recommended (Nugari and Salvadori, 2003), and it should be followed by tests *in situ* to evaluate the real situation, which is more complex, due to the involvement and intertwining of the many factors involved (Gorbushina *et al*., 2003). Biocide compositions and concentrations can

be assessed on microorganisms and stones in different conditions and their vitality can be measured with a variety of approaches (Favero-Longo *et al*., 2017).

The diffuse use of commercial chemical biocides has raised many concerns related to known dangers for human health (Caneva *et al*., 2008) and their ecotoxicity, particularly relevant when used in outdoor environment (Scheerer *et al*., 2009). This issue recently has drawn more and more attentions, and in the last two decades a new interest emerged for **natural compounds** with biological activities (Cappitelli and Villa, 2021) and **alternative treatments** (Ruffolo *et al*., 2017), leading the research towards alternative, less dangerous devitalization options (Fidanza and Caneva, 2019).

It is worth noting, however, that some authors are recently claiming that the natural origin of chemical compounds is not equal to the absence of toxicity (Cappitelli and Villa, 2021).

1.3. Aims

This PhD research delves into different applications of biological analyses to cultural heritage studies and conservation. The main focus is the investigation of outdoor stone surface biodeterioration, on natural and artificial lithic materials, in the field and under controlled laboratory conditions. Different areas of interest have been deepened to address understanding and prevention of biodeterioration processes. Adopted investigation techniques have been constantly based on conservative (sampling and modelling) approaches to minimally impact the original heritage materials.

Each one of the following chapters regards a different problem of biodeterioration of stone surfaces and deals with one or two of the macro-areas here listed:

A) influence of micro-environmental conditions on biodiversity;

B) physical and chemical space of interaction of lithobionts with their substrate;
C) overall effect of lithobiontic colonization on stone durability, meant as a balance between biodeterioration and bioprotection impacts;
D) efficacy of biocide treatments depending on selected substances, method of application and environmental conditions.

Outdoor cultural heritage can face variable micro-environmental conditions within a few (centi-)meters. These conditions influence lithobiontic community composition and, as a consequence, their

biodeteriogenic potential. For this reason, the comprehension of **micro-environmental factors impacting on biodiversity (A)** and the resulting **physical and chemical interaction of the communities with the substrate (B)** is particularly relevant, allowing to address conservation and prevention strategies. In particular, the definition of the most vulnerable areas could suggest the overriding concerns and the less urgent ones. In chapter 2 (*"Microenvironmental features drive the distribution of lichens in the House of the Ancient Hunt, Pompeii, Italy"*) this issue is considered with reference to lichen growth on stone surface in the context of a wide archaeological site.

Although in the last few decades many studies compared the patterns and processes of **microbial interactions with lithic material (B)**, the underlying mechanisms are often not clarified yet. For example, the widespread presence of meristematic microcolonial fungi on stone surfaces is a known problem, widely observed in all the environments, but the mechanism of their penetration within the rock still needs to be understood. A role of melanin analogous to that described for the penetration of pathogenic and phytopathogenic microcolonial fungi has been also hypothesised on stone, but never tested. To answer this scientific question, an *in vitro* protocol was set to study a model fungus penetration in presence or in absence of melanin (Chapter 3 - *"Hyphal morphology and substrate porosity -rather than melanization-drive penetration of black fungi into carbonate substrates"*). Artificial lithic substrates with different porosity conditions were assayed as standardized media to deepen the knowledge on penetration patterns of different fungal growth forms, suitable to improve conservation and prevention strategies.

Lithobiontic interactions may induce modifications in stone materials, impacting on their properties and, as a consequence, influencing their durability, which is a crucial problem in the field of conservation of cultural heritage. In particular, material properties related to durability, *e.g*. stone hardness, may be modified following different patterns and, as a consequence, the **overall effect of lithobiontic colonization on stone substrate (C)** can be thus very variable. This complexity, together with the limits in sampling, may be the reason for the scarce number of studies on this issue. Nevertheless, a comprehension of the contribution of lichens to biogeomorphology and the effective threat of lichens to conservation would be crucial to address choices of removing or preserving their colonization. In chapter 4 (*"Lichen impact on sandstone hardness as species-specific dependent"*), a standardised laboratory method is introduced and applied to study the impact of lichen species commonly found on outdoor sandstone surfaces, with the purpose of correlate stone hardness variations to specific **processes of physical and chemical interaction (B)** with the substrate.

Whenever microbial colonization effects move the balance between biodeterioration and bioprotection towards the more deteriorative outcome, or even if the colonization causes a discoloration which, without compromising stone integrity, modifies its colour or decipherability, biological patinas are usually removed. Biocidal treatments should always precede cleaning procedures and they are often applied with traditional procedures and substances, without considering possible variations related to material, organisms or external conditions. Chapter 5 (*"The application protocol impacts the effectiveness of biocides against lichens"*) discusses *in situ* devitalization tests, carried on in an archaeological site target of a programmed

cleaning intervention, to calibrate methods of application of biocidal substances against lichens in order to unveil the most effective technique. In particular, the work innovatively examines the importance of different pre- and post-treatment procedures and the differential permanence of biocidal substances within substrates. A further research contribution dealing with the hypothesis that allelopathic secondary metabolites of lichens may be considered as alternative green biocides against microbial communities on stonework is additionally reported in Appendix 1.

As an additional appendix, this PhD thesis includes preliminary insights on an archaeo-microbiology investigation carried out in the context of a multi-and inter-disciplinary collection of scientific data, including biological readings, to answer archaeological questions (project "BEyond ARCHAEOlogy: an advanced approach linking East to West through science, field archaeology, interactive museum experiences", Horizon 2020-Marie Curie-RISE 2019-2022). In particular, Appendix 2 reports and preliminary discusses molecular analyses carried out on soil fungal communities to examine biodiversity and to suggest possible interpretation of archaeological layers in a burial excavation in the Okayama Prefecture, Japan.

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Preface

The influence of climatic factors on the biodiversity of lichens and other lithobiontic organisms has long been confirmed, but in the field of outdoor stone cultural heritage the issue is further complicated by the potential influence of other (micro-)environmental features.

With particular focus on lichens, this work considers lithobiontic colonization in the archaeological site of Pompeii (Naples, Italy), exposed to atmospheric agents and tourist transit. The House of the Ancient Hunt (*Casa della Caccia Antica*) is a *domus* which was subject, from 2016 to 2019, to interdisciplinary studies aimed to deepen the knowledge of the structure and the state of deterioration of its surfaces [project: «Da Pompei a Venaria. Per un progetto di conoscenza, valorizzazione, divulgazione: la Domus della Caccia Antica», funded by Fondazione CRT (Richieste Ordinarie 2016-II: 2016.2408) as a side project of the wider triennal collaboration project «Tra Pompei e Venaria: Progetti per la conservazione e il restauro» between Soprintendenza speciale per Pompei Ercolano e Stabia (MiBACT), University of Torino-S.U.S.C.O.R. and Centro Conservazione e Restauro «La Venaria Reale»]. A focus on biodeterioration in this context is particularly valuable, and together with chemical and physical analyses is necessary to address suitable conservation strategies. The research outcomes and their consequences can be easily transposed to the other spaces of the large archaeological area of Pompeii, constantly exposed to biodeterioration agents due to its extension and its nature of outdoor archaeological site.

In the House of the Ancient Hunt, on natural and artificial lithic surfaces, dense lichen communities were detected and identified with non-destructive micro-sampling. The communities were heterogeneous in cover and species composition, leading to the hypothesis of micro-

environmental features as main cause of variability and drivers of different biodeterioration processes. The biodiversity as a function of estimated variables (material, related to porosity; aspect, related to aridity; vertical distance from the ground, related to capillary water rise; horizontal distance from the nearest corner, related to humidity stagnation; room dimension, related to ventilation and vegetative particles transportation) was related to the physical and chemical biodeterioration caused by the more frequent species. In particular, hyphal penetration within the substrate and the production of metabolites known for their biodeteriogenic activity were characterized. These last analyses were realized exclusively on already detached and de-contextualised fragments, in order to maintain a conservative approach.

Chapter 2

Microenvironmental features drive the distribution of lichens in the House of the Ancient Hunt, Pompeii, Italy

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Abstract

On the stone cultural heritage, the influence of architecture-related microenvironmental features on lichen diversity, abundance and consequent threats for conservation has been still poorly characterized to support management plans. Such relationships were here investigated on the vertical surfaces of the House of the Ancient Hunt in Pompeii, archaeological site in S-Italy where the variability of lichen saxicolous communities has been still completely neglected despite their widespread occurrence. Lichen colonization in semiconfined rooms was sporadic and limited to *Dirina massiliensis*, while a remarkable turnover of six communities, encompassing 22 species, characterized mortar, painted and plastered surfaces in outdoor environments, with local covers up to 80%. Microscopic and spectroscopic analyses displayed the deteriogenic potential of three dominant species, due to hyphal penetration within paint and plaster layers (*Verrucaria macrostoma*) and

the release of oxalic acid and/or secondary metabolites with acidic and chelating functions (*D. massiliensis, Lepraria lobificans*). A higher vertical distance of surfaces from the ground and a larger room dimension were the main conditional factors related to a higher lichen abundance and the distribution of the different communities. Such knowledge on architecture-related microenvironmental features driving lichen distribution and biodeterioration threats may contribute to address restoration priorities and conservation strategies.

Keywords
Archaeological areas, Biodeterioration, Community variability, Environmental factors, Lichens, Stone cultural heritage

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2.1. Introduction

The growth of lithobiontic communities on the stone cultural heritage depends on complex relationships among (micro-)organisms, materials and the environment (Pinna, 2017). Physical and chemical properties of a stone substrate determine its bioreceptivity, i.e. its attitude to be colonized by one or several groups of living organisms (Guillitte, 1995; Miller *et al*., 2012). However, patterns of biological diversity and abundance on a certain material vary with its geographic location, related to, e.g., macroclimate and pollution, and may further depend on local factors determining distinct microniches (Caneva and Ceschin, 2008; Pinna, 2017). Parameters controlling microbial growths, as light intensity,

water availability, and temperature, relate to regional climate conditions, but also to extrinsic and intrinsic features of each stone surface, as aspect, shading rates, ventilation, vertical distance from the ground and other properties related to architectural geometries (Cutler *et al*., 2013; Caneva *et al*., 2015; Ahmad, 2015). Such local factors affect the suitability of each surface to be colonized by a certain lithobiontic species -depending on its autoecological requirements-, but they may also influence the external propagule supply which triggers colonization (De Nuntiis *et al*., 2003).

In the case of lichens, biodeteriogens on a wide spectrum of stone surfaces, the presence of different species was related to biogeophysical and biogeochemical processes with diverse deterioration impact (Gazzano *et al*., 2009; Salvadori and Casanova-Municchia, 2016; Seaward, 2015). With this regard, for different materials and climatic areas, the understanding of factors controlling colonization patterns of lichen species may guide the identification of dangerous microclimatic conditions for stone conservation and the definition of restoration priorities. In the case of stone monuments in tropical area, the forest canopy gradient was related to different lichen-dominated communities, with different degrees of aggressiveness (Caneva *et al*., 2015). In the Mediterranean region, the variability of epilithic communities with respect to gradients of environmental variables was examined on natural rock outcrops, highlighting the importance of solar radiation and water availability at the micro-scale (Giordani *et al*., 2014). The rich literature on biodeterioration in cultural heritage sites along the Mediterranean basin highlights a high level of lichen diversity and a wide range of lichen-related biodeterioration issues (Nimis *et al*., 1987; Piervittori, 2004; Seaward, 2015 with refs. therein). The influence of environmental parameters on lichen diversity in monumental areas was also remarked

(e.g. Ariño *et al*., 1995; Nimis *et al*., 1998; Nascimbene and Salvadori, 2008; McIlroy de la Rosa *et al*., 2013). However, relationships between architecture-related microenvironmental factors, lichen diversity and abundance, and consequent deterioration threats have been still poorly supported with numerical analyses.

Surprisingly, at the best of our knowledge, lichens have been quite completely neglected in studies on ancient Pompeii, one of the most important archaeological sites in the world, although lichen occurrence is evident on many natural and artificial stone materials (*sensu* Caneva *et al*., 2008), affecting their aesthetic value and potentially threatening their conservation (Fig. 1A-D). Information has been recently provided on the influence of regional-scale climatic factors on the presence/distribution of biodeterioration phenomena in the archaeological area (Traversetti *et al*., 2018). However, knowledge on lichen diversity, species distribution, and related deterioration issues is still lacking.

In this paper, we characterized lichen diversity and distribution on masonries and wall paintings of the Pompeian House of the Ancient Hunt (*Casa della caccia antica*). The study aims (a) to verify if the lichen presence is homogeneously distributed through the different rooms of the House, and (b) to test the hypothesis that the distribution patterns of lichen species are related to different materials, aspect and other architecture-related features of stone surfaces, considered as proxies of different microclimatic conditions. Deterioration patterns related to dominant lichen species are also preliminary assayed and discussed with regard to their significance for conservation.

2.2. Materials and Methods

2.2.1. Investigation site

Since its first excavations, the ancient city of Pompeii has begun to display a wide variety of conservation problems, which are particularly difficult to control also due to the extension of the archaeological area (more than 66 ha), the massive tourist presence, management difficulties, and uncautious or outmoded restoration operations (Wollner, 2013). Since 2012, the *Grande Progetto Pompei* aims to enhance the effectiveness of the activities for protecting the archaeological area and to address a transition from extraordinary interventions to a continuous and planned conservation maintenance (Osanna and Rinaldi, 2018). In the framework of this and related side projects, several Pompeian monuments were and still are object of restoration and supportive diagnostic investigations.

The House of the Ancient Hunt is now the focus of a two year project (*Da Pompei a Venaria. Per un progetto di conoscenza, valorizzazione, divulgazione: la Casa della Caccia antica*, directed by D. Elia and supported by Fondazione CRT, Italy) which aims at a systematic reappraisal of the *domus* in a multisciplinary perspective (Elia and Meirano, 2018). Stratigraphical verifications, archaeological investigations and studies on building techniques have been made in order to achieve a better knowledge and to allow for revisited interpretations of the phases which characterized the long life of the *domus*. An intensive diagnostic program involving chemists, physicists, geologists, botanists, etc. has been launched aiming at the recognition of building and decoration materials and at supporting conservation operations. Meanwhile, a selection of the wall paintings and a mosaic are the object of practical activities of the Master Degree in Conservation

and Restoration of Cultural Heritage of the University of Torino, in agreement with the Foundation Centre for Conservation and Restoration of Cultural Heritage "La Venaria Reale".

The House (VII.4.48) is a *domus* of approximately 600m^2 (Allison and Sear, 2002) at less than 200m from the Pompeii *forum*. It was built around the middle of the II century BC and its internal organization was modified in the various phases of use. The House displays an *atrium* and a *peristylium* surrounded by several rooms, distinguishable as outdoor or semi-confined environments for the presence/absence of modern protective roofs, for a total of 24 rooms (Fig. 1E).

The House was excavated between 1833 and 1835. Around the middle of the XIX century, some rooms were covered with sloping roofs in tiles and wood. In the 40s-50s of the XX century, rooms 4 and 15 were covered with roofs in concrete. Interventions of wall integration were led in 1978. The last consolidations were performed between 2009 and 2010. Nowadays, the House displays a wide range of materials, environmental and conservation conditions. In semi-confined environments, wall paintings, plaster of the preparation layer (*arriccio*) and mortar between stone blocks are often well conserved, while in outdoor environments they are generally more deteriorated, with detachments, swelling, discoloration, lichens and biofilms containing cyanobacteria and, subordinately, microcolonial fungi and green algae. Stone blocks of Sarno limestone, volcanic and pyroclastic rocks, composing the structure of ancient walls and modern integrations, are well preserved, with their surface poorly characterized by lithobiontic communities. In most of the rooms, the floor is covered with soil for protective purposes, leading the presence of spontaneous vascular plants.

Fig. 1. Lichen colonization on different stone substrates of the ruins of Pompeii (Italy). (A) Tuff blocks of an opus reticulatum *near the* Antiquarium*. (B) Carbonatic rock slates and terracotta jars in a* thermopolium*. (C) Millstones in the* pistrinum *(bakery) of Popidius Priscus. (D) Corner between Via della Fortuna and Vicolo Storto. (E-I) House of the Ancient Hunt: map (E; semi-confined rooms are crossed; numbers of rooms with maximum lichen cover higher than 1% are circled; scale bar: 5 m) and painted surfaces in the outdoor environment of room 12 (F; relevés on the SSE and ENE-facing walls in G and H, respectively) and in the semi-confined room 6 (I). Asterisks highlight the localization of lichen communities.*

2.2.2. Sampling design and environmental parameters

Lichen presence/absence was surveyed on the vertical stone surfaces of the 24 rooms of the House, including: (a) ancient Roman and (b) modern mortars binding the tuff and limestone blocks, (c) plastered and (d) painted surfaces. Observations were run with the aid of a handlens in June 2017 from the ground level up to 3.5m in height, while higher levels (where present) were only visually observed from the distance. The maximum lichen cover (%) within each room was evaluated by visually surveying a single 50 × 50 cm plot, selectively placed on the most colonized surface.

Diversity relevés were performed through the rooms where lichens occurred with a maximum cover higher than 1% (Fig. 1E-H), with the exception of rooms 16 and 17, where most of lichen colonization was located at a height higher than 3.5 m and was not accessible for detailed observations and sampling. In these rooms (n = 10), independent 50 × 50 cm plots were preferentially distributed to represent the maximum colonization per material (a-d) per aspect. Each plot was surveyed using a square grid divided into 25 quadrats (10 × 10 cm). The cover of lichen species within each plot and their presence within each quadrat were estimated visually. The frequency of each species within each plot was calculated as the sum of their occurrences within the grid quadrats. Lichens were identified using Clauzade and Roux (1985), Smith *et al*. (2009), McCune (2016) and monographic descriptions. Nomenclature follows Nimis (2016). Sample vouchers were deposited at the Cryptogamic Herbarium of the University of Torino (HB-TO Cryptogamia).

For each quadrat, beside the material (MAT) and the aspect (ASP), the following features were evaluated, categorized and indexed: vertical distance from the ground (HEI), horizontal distance from the nearest wall corner (DNC), room dimension (ROD). Materials were categorized on the

basis of different grain size (see Piovesan *et al*., 2009) -related to porosity and water retention-, as follows: ancient Roman mortars (4), modern mortars (3), plastered surfaces (2), painted surfaces (1). Rock blocks of ancient and modern walls, on which the colonization was subordinated to and driven by that of the ancient and modern binding mortars, respectively, were not separately categorized, but considered with these latter. Aspect, related to aridity, was categorized as follows (modified from Pharo *et al*., 1999): SSE (4), WSW (3), ENE (2), NNW (1). The vertical distance from the ground -related to capillary water rise (Hall and Hoff, 2007)- was categorized as follows: ≤ 50 cm (1), 51-150 cm (2), 151-250 cm (3), > 250 cm (4). The horizontal distance from the nearest corner -related to humidity stagnation (Abuku *et al*., 2009)- was categorized as follows: ≤ 50 cm (1), 51-100 cm (2), ≥ 101 cm (3). The room dimension -related to ventilation (Zhang and Chen, 2006)- was categorized on the basis of the floor area as follows: < 5 m^2 (1; rooms 5a, 5c), 5-10 m^2 (2; rooms 1, 9, 21); 11-35 m^2 (3; rooms 7, 11, 14), > 35 m^2 (4; room 12).

2.2.3. Statistics

The relative importance of components of γ-diversity [i.e. similarity (S), relativised richness difference (D), and relativised species replacement (R)] was evaluated for all combinations of plots through the overall rooms and for each separate room by analysing the matrix of species presence/absence with SDR Simplex software (2001) using the Simplex method (SDR Simplex; Podani and Schmera, 2011). Similarity (S) was calculated following the Jaccard coefficient of similarity:

$$S_{jac} = a/n$$

where *a* is the number of species shared by the two plots, and *n* is the total number of species.

The relativised richness difference (D) was calculated as the ratio of the absolute difference between the species numbers of each site (*b*, *c*) and the total number of species, *n*:

$$D = |b\text{-}c|/n$$

Relativised species replacement (R) was calculated as:

$$R = 2 * \min\{b, c\}/n$$

A relativised β-diversity as the sum of R + D, a relativised richness agreement as the sum of R + S, and a relativised nestedness as the sum of S + D were also calculated for each pair of areas following Podani and Schmera (2011).

A first Principal Coordinate Analysis (PCoA-I; symmetric scaling with species score divided by standard deviation, centring samples by samples, centring species by species; Ter Braak and Šmilauer, 2002) was performed on the matrix of species frequencies at the plot level to visualize the relatedeness of communities through the House. A second ordination of plots by Principal Coordinate Analysis (PCoA-II) was based on a matrix including microenvironmental features (HEI and DNC of the central quadrat of each plot, prevalent MAT for each plot, ROD, ASP) and overall lichen abundance (LICH, as total of specific lichen frequecies per plot) to visualize their correlation.

Quadrats 10 × 10 cm were classified (UPGMA, Sokal-Sneath as dissimilarity coefficient, arbitrary resolution of ties; Podani, 2001) on the basis of species presence/absence. The matrices of species presence/absence and microenvironmental features at the quadrat level were processed through a Canonical Correspondence Analysis (CCA), which partitions variation explained by each variable and constructs a model of significant variables (CCA using biplot scaling for interspecies

distances, Hill's scaling for inter-sample distances; choosing forward selection of variables option; performing Monte Carlo permutation test on the first and all ordination axes) (Ter Braak and Verdonschot, 1995).

Classification analyses were performed using SYN-TAX 2000 - Hierarchical Classification (Podani, 2001), while ordinations were performed using CANOCO 4.5 (Ter Braak and Šmilauer, 2002).

2.2.4. Spectroscopic, chromatographic and microscopic analyses

Millimetric fragments of thalli of dominant species in the House (*Verrucaria macrostoma*, *Dirina massiliensis*, *Lepraria lobificans*) were collected using lancets and inoculation needles, without affecting the colonized substrate, for performing analyses on the lichen potential biodeteriogenic activity (Gazzano *et al*., 2009). In particular, (i) the production of metabolites chemically affecting mineral stability through acidolytic or chelating actions, as oxalic acid and secondary metabolites, and (ii) the hyphal penetration through the substrate, acting physical disaggregation, were evaluated.

Oxalic acid production was assessed with reference to the occurrence of oxalate deposits in the thalli, evaluated by μ-Raman spectroscopy. Raman spectra were collected with a micro-spectrometer Horiba Jobin Yvon HR800 equipped with an HeNe laser at an excitation wavelength of 632.8 nm, a CCD air-cooled detector operating at −70° C, and an Olympus BX41 light microscope. The spectra were compared with oxalate spectra reported by Edwards *et al*. (2003).

The production of secondary metabolites was qualitatively evaluated by Thin Layer Chromatography (TLC). At least three specimens per species were extracted with acetone. Silicagel SIL G-25 UV254 (Macherey-Nagel; Düren, Germany) was used as the support on glass

plates and a solution of toluene and acetic acid (170:30) was used as the solvent ('Solvent C' *sensu* Orange *et al*., 2010) for compound separation. The developed chromatograms were examined using a Spectroline Longlife UV lamp (254 and 365 nm wavelengths; Spectronics Corporation, Westbury, NY, USA) with fluorescent analysis cabinet (without spray reagents). The Retention factors (Rf) of the observed spots were defined in relation to reference compounds [i.e. norstictic acid extracted from *Pleurosticta acetabulum* (Neck.) Elix and Lumbsch and usnic acid produced by Sigma-Aldrich (St Louis, MO, USA)].

Finally, one fragment of painted surface (20×10×5 mm) colonized by *V. macrostoma*, already detached and lying out of context on the ground of room 12, was used to examine the lichen-substrate interface under reflected light (RLM) and scanning electron (SEM) microscopy. RLM observations were carried out using an Olympus SZH10 on a cross section stained using the periodic acid - Schiff (PAS) to visualize the biological component within the lithic substrate (Favero-Longo *et al*., 2005). The hyphal penetration component (*sensu* Favero-Longo *et al*., 2005) was characterized in terms of structural organization and depth of penetration through the paint and plaster layers. SEM observations were carried out with a scanning electron microscope JEOL JSM IT300LV in the secondary electon mode (High Vacuum – Low Vacuum 10/650 Pa - 0.3-30 kV) on a second carbon-coated cross section. The cross-sections are conserved in the Lichen-Petrographic Collection of the Herbarium of the University of Torino (Gazzano *et al*., 2007).

2.3. Results

2.3.1. Lichen diversity and distribution

Lichen colonization characterized 12 out of the 24 rooms of the House (Fig. 1E). Eleven out of seventeen outdoor environments (65%) displayed maximum lichen cover higher than 1%, with values ranging from 2 to 80% (Fig. 1F-H and 2A). Few thalli sparsely occurred in the remnant outdoor environments (maximum cover < 1%), appearing negligible for conservation issues. Only one of the seven semi-confined environments displayed colonized surfaces (12% max. cover in room 6; Fig. 1I).

Twenty-two lichen species were found through 52 out of the 75 plots in the 10 rooms surveyed in detail (Table 1), where 31% of plots was instead uncolonized. Diversity in outdoor rooms ranged from 11 to 3 species, with maximum species diversity per plot varying from 5 to 1, while only *Dirina massiliensis* colonized the back wall of the semi-confined room 6 (Fig. 1I). This latter species was the most widespread through the House, occurring in 80% of rooms, followed by *Verrucaria macrostoma* (70%) and *Lepraria lobificans* (70%). These three species also displayed both the highest frequencies per plot (> 20%) and per quadrat (> 8%), and their cover values were higher than 1% in at least 10% of plots (Fig. 2B, left side). *V. macrostoma* and *D. massiliensis*, in particular, displayed maximum cover values of 80% and 55%, respectively. Other seven species only locally (< 3% of plots) displayed remarkable covers up to 10% (Fig. 2B, centre), while remnant species only punctually occurred (Fig. 2B, right side).

The evaluation of the relative importance of components of γ-diversity, i.e., S, D and R, for all pairs of plots showed a low species similarity through the House (S = 15.8%), whereas the species

replacement was the major component (R = 52.8%). Relativised β-diversity (R + D) was 84.2% (Table 2). The SDR analyses performed separately for each room also showed relatively low similarity (av. S = 21.5%) and high species replacement (av. R = 48.7%), with the exception of room 5a and the semi-confined room 6, where two and one species only occurred, respectively. Accordingly, in Principal Coordinate Analysis-I (PCoA-I), which explained 84.9% of total variance of species frequency values, plots of each room were sparsely distributed through the diagram (Fig. S1 and Table S1a).

PCoA-II (Fig. 3A), which ordinated plots on the basis of microenvironmental features and the overall lichen abundance (LICH, as total of specific lichen frequencies) explained 89.1% of total variance (details in Table S1b). LICH was positively correlated, along the first axis (35.8%), with ROD and, subordinately, HEI. Plots with no lichens (n = 23) clustered in the left of the diagram, characterized by higher values of MAT and ASP.

The classification of quadrats on the basis of the species presence resulted in the separation of six main groups (*i-vi*), characterized by the combination of the three dominant species (*V. macrostoma*, *L. lobificans*, *D. massiliensis*) with one or more subordinate species (Table S2; Fig. S2). The Canonical Correspondence Analysis (CCA; Fig. 3B and C) displays the ordination of clusters *i-vi* with respect to species and microenvironmental features at the quadrat scale. The analysis extracted four main axes which explained 93.0% of species-environmental relationships. The first and all canonical axes were significant (Monte Carlo test, P-value = 0.002). All microenvironmental features exhibited significant conditional effect according to forward selection ($P < 0.002$; details in Table S3). The first (41.3% of species-environmental correlation) and second (24.2%) axes were characterized by HEI (weighted correlation, w.c., -0.85 with axis 1) and ROD, (w.c. -0.72 with axis 2),

respectively, which displayed the higher conditional effects (F-value: 21.2 and 14.2, respectively). MAT, mainly correlated with axis 3 (w.c. 0.75), and the other microenvironmental factors exhibited lower conditional effects (F-values: MAT 9.18, ASP 7.94, DNC 7.30). The three dominant species separately scattered in the diagram, with *V. macrostoma* and *D. massiliensis* positively and negatively correlated with HEI, respectively, and *L. lobificans* negatively correlated with ROD and positively with MAT. In quadrats of cluster *i* (n = 242), characterizing the left side of the diagram (higher height from the ground, dry exposition, far from the humid corners, larger room dimension, finegrained materials: painted and plastered walls), *V. macrostoma* occurred alone and associated with *Flavoplaca citrina* or *Candelariella aurella*, both sharing its photophytism, xerophytism and tolerance of high eutrophication (Nimis, 2016), and/or *D. massiliensis*. The presence of this latter species alone or associated with species less tolerant of eutrophication (*L. lobificans*, *Verrucaria muralis*, *Xanthocarpia lactea*, *Pyrenodesmia chalybeia*, *Aspicilia calcarea*, *Protoblastenia incrustans*) characterized cluster *ii* (n = 127), positively correlated with axis 1. Clusters *iii* (n = 69) and *iv* (n = 24), characterized by *Flavoplaca coronata* and *V. muralis*, respectively, alone or in association with the three dominant and other species, scattered in the centre of the diagram. Clusters *v* (n = 146) and *vi* (n = 16) were characterized by *L. lobificans* alone and associated with other species sharing mesophytism and poor tolerance of eutrophication (*v*: *Thelidium incavatum*, *Scytinium* sp., *X. lactea*; *vi*: *Strigula calcarea*; quadrats scattered in the right side) or with *V. macrostoma* (quadrats scattered in the left side). Basal branches of the classification represented scattered occurrence of rare species, here considered as cluster *vii* (n = 22).

Table 1
Lichen diversity and abundance in the House of the Ancient Hunt.

	Rooms										
	12	14	21	7	1	11	5a	9	5c		6
Plots per room (n)	12	5	5	6	9	14	12	5	4		3
Max. total cover per plot (%)	80.2	80.0	30.0	12.4	12.2	8.0	8.0	5.1	2.3		2.0
Species per room (n)	11	4	3	11	5	10	3	7	4		1
Max. species per plot (n)	4	2	2	5	3	5	1	4	4		1
Av. species per plot (n)	2.3	1.6	0.8	2.5	1.1	1.7	0.4	1.4	1.3		1.0

Species		Colonized rooms (n out of 10)	Max. species cover (%)	Cover ≥1% (% of plots)	Frequency per plot (%)	Frequency per quadrat (%)	Max. species cover per room (%)									
							12	14	21	7	1	11	5a	9	5c	6
Verrucaria macrostoma DC.	Vm	7	80.0	18.7	28.00	14.13	70.0	80.0	0.1	12.0	12.0	6.0	–	0.1	–	–
Dirina massiliensis Durieu & Mont.	Dm	8	55.0	12.0	28.00	12.05	55.0	0.1	30.0	–	0.1	0.1	1.0	0.1	–	2.0
Lepraria lobificans Nyl.	Lm	7	8.0	13.3	20.00	8.21	1.0	–	2.0	6.0	–	2.0	8.0	0.1	2.0	–
Flavoplaca coronata (Körb.) Arup, Frödén & Søchting	Fo	5	5.0	1.3	12.00	4.05	0.1	0.1	–	0.1	–	0.1	–	5.0	–	–
Flavoplaca citrina (Hoffm.) Arup, Frödén & Søchting	Fc	3	1.0	1.3	6.67	2.19	1.0	–	–	–	–	0.1	–	0.1	–	–
Arthonia calcarea (Sm.) Ertz & Diederich	Ac	3	10.0	2.7	4.00	1.71	4.0	–	–	10.0	–	–	–	–	0.1	–
Verrucaria muralis Ach.	Vu	5	8.0	2.7	10.67	1.49	0.1	–	–	8.0	–	2.0	–	0.1	0.1	–
Thelidium incavatum Mudd	Ti	2	6.0	1.3	4.00	1.17	–	–	–	0.1	–	–	6.0	–	–	–
Lecidella cfr. *asema* (Nyl.) Knoph & Hertel var. *asema*	La	2	5.0	1.3	4.00	0.85	5.0	–	–	–	–	0.1	–	–	–	–
Strigula calcarea Bricaud & Cl. Roux	Sc	2	10.0	2.7	4.00	1.01	–	10.0	–	4.0	–	–	–	–	–	–
Candelariella aurella (Hoffm.) Zahlbr.	Ca	2	0.1	–	4.00	0.37	–	–	–	–	0.1	0.1	–	–	–	–
Toninia aromatica (Sm.) A. Massal.	Ta	1	0.1	–	1.33	0.37	–	–	–	0.1	–	–	–	–	–	–
Myriolecis albescens (Hoffm.) Sliwa, Zhao Xin & Lumbsch	My	2	0.1	–	4.00	0.27	–	–	–	0.1	0.1	–	–	–	–	–
Xanthocarpia lactea (A. Massal.) A. Massal.	Xl	2	0.1	–	2.67	0.21	–	–	–	–	–	0.1	–	0.1	–	–
Pyrenodesmia chalybaea (Fr.) A. Massal.	Pc	1	0.1	–	1.33	0.16	–	–	–	–	0.1	–	–	–	–	–
White squamulose R-	Ws	1	0.1	–	1.33	0.11	–	–	–	–	–	–	–	–	0.1	–
Calogaya pusilla (A. Massal.) Arup, Frödén & Søchting	Cp	1	0.1	–	1.33	0.05	0.1	–	–	–	–	–	–	–	–	–
Scytinium sp.	Sy	1	0.1	–	1.33	0.05	–	–	–	0.1	–	–	–	–	–	–
Acarospora fuscata (Schrad.) Arnold	Af	1	0.1	–	1.33	0.05	–	–	–	0.1	–	–	–	–	–	–
Catapyrenium cfr. *daedaleum* (Kremp.) Stein	Cd	1	0.1	–	1.33	0.05	0.1	–	–	–	–	–	–	–	–	–
Protoblastenia cfr. *incrustans* (DC.) J. Steiner	Pi	1	0.1	–	1.33	0.05	–	–	–	–	–	0.1	–	–	–	–
Lecania sylvestris(Arnold) Arnold var. *sylvestris*	Ls	1	0.1	–	1.33	0.05	0.1	–	–	–	–	–	–	–	–	–

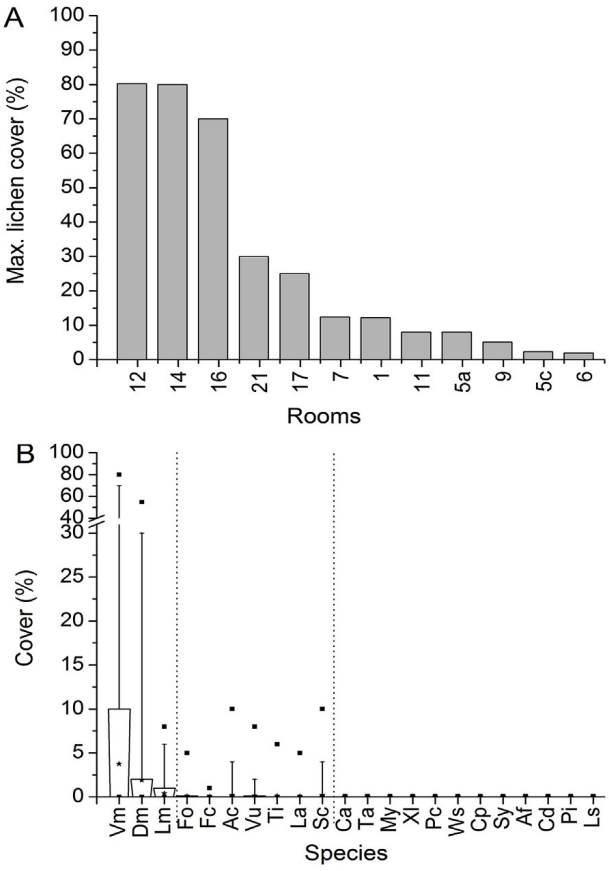

Fig. 2. Lichen colonization through the House. (A) Maximum lichen cover in the rooms with maximum cover value higher than 1%. (B) Ranges of species cover (%): dominant species (left), locally abundant species (centre), punctually occurring species (right). Maximum (■), 99th percentile (upper whisker), 75th percentile (top box), average (star) cover. Abbreviations of species names in Table 1.

Table 2. Percentage contribution from the SDR Simplex analyses of lichen communities through the overall rooms and in each room. S (relative similarity), R (relative replacement), D (relative richness difference), R + D (relative β-diversity), S + R (relative richness agreement), S + D (relative nestedness).

Rooms	S	D	R	R + D	S + R	S + D (-anti nestedness)	Matrix fill
All	15.8	31.5	52.8	84.2	68.5	31.6	9.4
12	18.5	31.0	50.5	81.5	69.0	37.1	20.5
14	20.0	23.3	56.7	80.0	76.7	30.0	40.0
21	33.3	22.2	44.4	66.7	77.8	33.3	44.4
7	10.3	38.8	50.8	89.7	61.2	22.7	27.3
1	30.8	27.5	41.7	69.2	72.5	55.0	40.0
11	15.7	36.4	47.8	84.3	63.6	39.3	26.7
5a	20.0	0.0	80.0	80.0	100.0	20.0	25.0
9	0.0	34.4	65.6	100.0	65.6	0.0	33.3
5c	25.0	75.0	0.0	75.0	25.0	100.0	62.5
6	100.0	0.0	0.0	0.0	100.0	100.0	100.0
Av. all rooms	21.5	29.9	48.7	78.5	70.1	36.2	33.1
Av. outdoor rooms	19.3	32.1	48.6	80.7	67.9	37.5	35.5

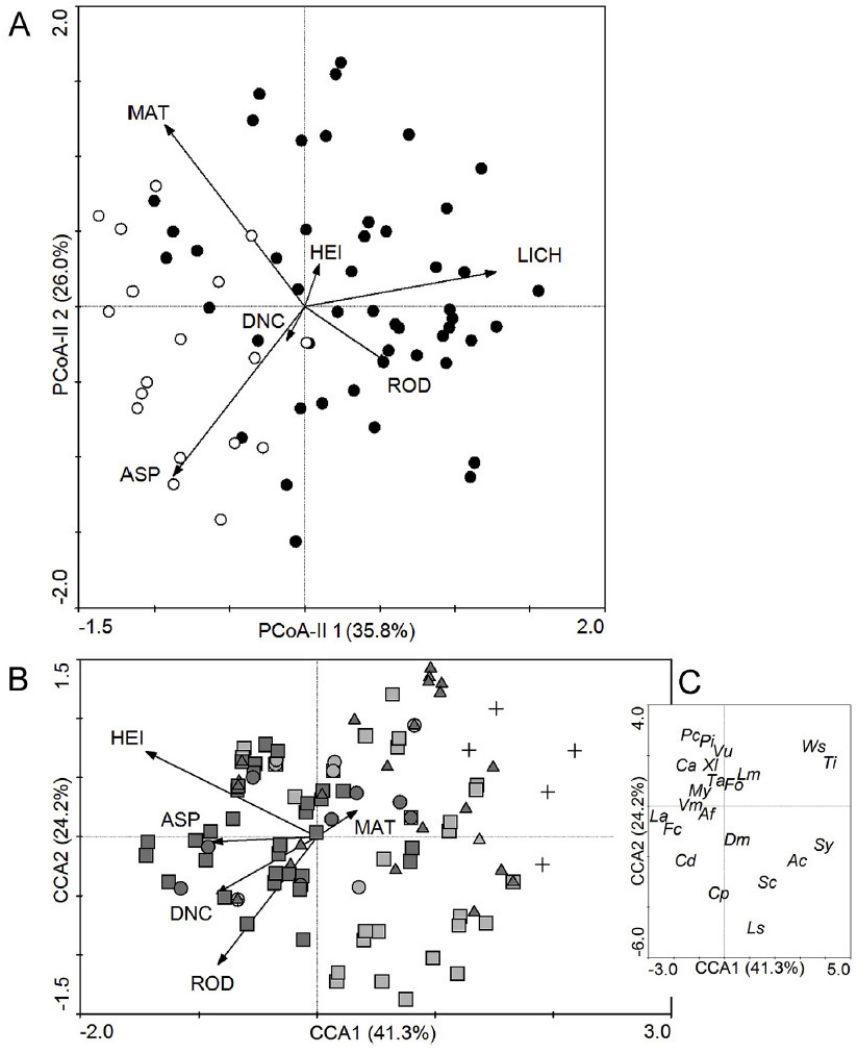

Fig. 3. Relationships between lichen colonization and microenvironmental features. (A) PCoA-II: ordination of plots on the basis of their microenvironmental features (dominant material, MAT; aspect, ASP; vertical distance from the ground, HEI; horizontal distance from the nearest wall corner, DNC; room dimension, ROD) and the overall lichen abundance (LICH). Black and white dots indicate plot with and without lichens, respectively (PcoA-II scores in Table S1B). (B-C) Factorial maps in the Canonical Correspondence Analysis (CCA) showing (B) the position of quadrats (symbols according to UPGMA classification in Fig. S1: i*, dark grey square;* ii*, light grey square;* iii*, dark grey circle;* iv*, light grey circle;* v*, dark grey triangle;* vi*, light grey triangle;* vii*, cross) together with the contributions of microenvironmental features and (C) of different species (abbr. in Table 1). All the extracted axes displayed in the figure were significant according to Monte Carlo test (CCA scores in Table S3).*

2.3.2. Lichen potential deterioration activity

Raman spectroscopy displayed spectra attributable to calcium oxalate dihydrate ($CaC_2O_4 \cdot 2H_2O$) within the thalli of *D. massiliensis* (Fig. S3): bands with wavenumber at 1476, 910 and 504 cm^{-1} were assignable to $\nu(CO_2)$ sym, $\nu(CC)$ and $\delta(CO_2)$ sym vibrational modes, respectively (Edwards *et al*., 2003). Similar spectra were not detected in correspondence of *L. lobificans* and *V. macrostoma*.

TLC on *D. massiliensis* displayed the occurrence of erythrin ($C_{20}H_{22}O_{11}$), also preliminary detected with spot tests (C+ red; *sensu* Orange *et al*., 2010), and other unidentified substances (see Smith *et al*., 2009). In *L. lobificans*, the occurrence of atranorin ($C_{19}H_{18}O_8$), stictic ($C_{19}H_{14}O_9$), constictic ($C_{19}H_{14}O_{10}$) and (±) roccellic ($C_{17}H_{32}O_4$) acids was detected. No secondary metabolites were found in *V. macrostoma* (data not shown).

On the other hand, *V. macrostoma* displayed a remarkable hyphal penetration within the substrate. RLM (Fig. 4A-D) displayed the continuous presence of a network of hyphae and hyphal bundles (diameter up to 40 µm) through the paint layer and the upper, fine part of plaster, down to 1.0 mm. Hyphal penetration also sparcely affected the deeper part of plaster, with maximum penetration down to 2.0-2.5 mm. SEM observations indicated both the micron-scale porosity of mortar matrix (Fig. 4E and F) and the boundaries of sub-millimetric clasts (Fig. S4) as passageways for the hyphal growth.

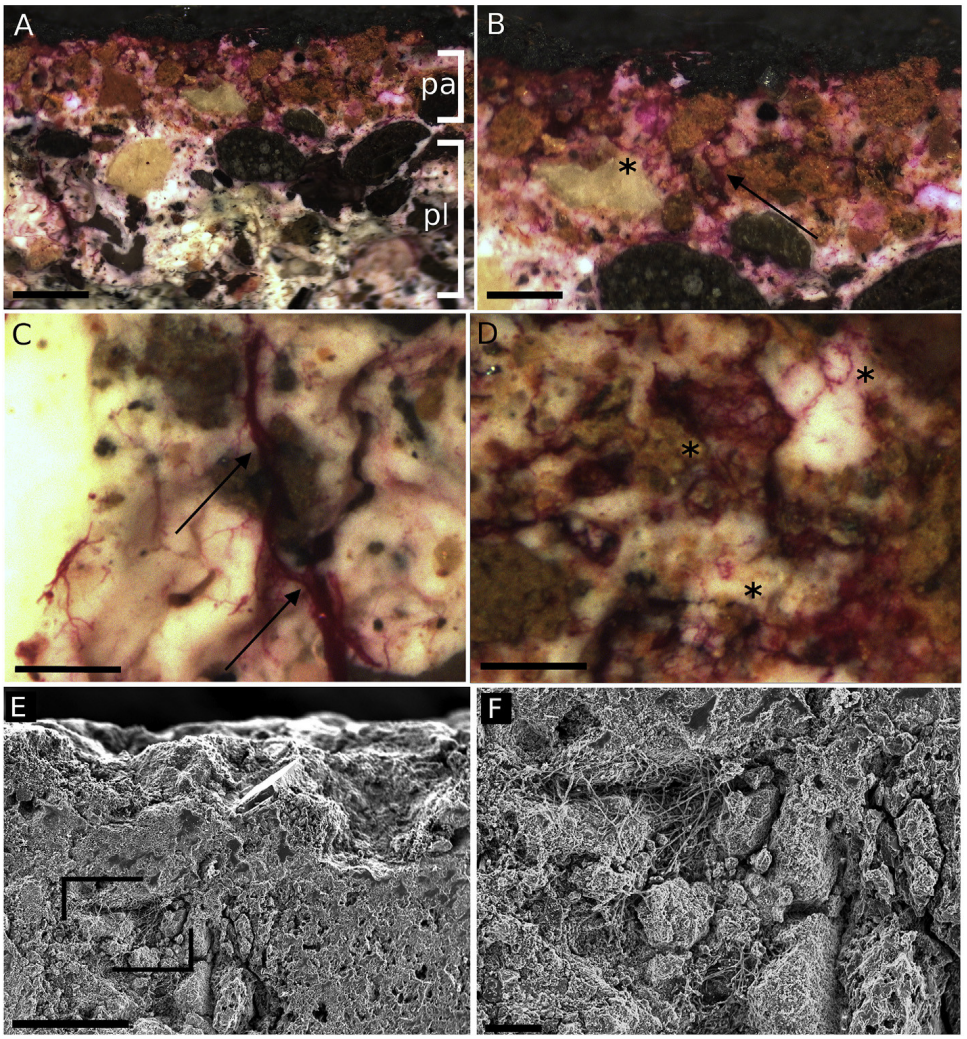

Fig. 4. Hyphal penetration component of Verrucaria macrostoma *within the paint and plaster layers (A-D: cross section stained by PAS and observed by RLM; E and F: cross section observed by SEM). (A) Overview of hyphal penetration through the different layers (pa, paint layer; pl, plaster layer). (B) Magnification of the network of hyphal bundles (arrow) and hyphae (asterisks) at the paint layer. (C) Magnification of hyphal bundles (arrows) in the upper part of the plaster layer. (D) Network of hyphae (asterisks) growing through the mortar matrix. (E, and magnification in F) Hyphal growth through the porosity of the mortar matrix. Scale bars: 1mm (A), 500 µm (B, E), 250 µm (C, D), 100 µm (F).*

2.4. Discussion

Although lithobiontic communities are remarkable threats for the conservation of cultural heritage, scientific knowledge on biodeteriogens in the archaeological area of Pompeii is limited to reports on vascular plants (Ciarallo and D'Amora, 1990) and few investigations on microbial patinas of fungi and bacteria, responsible for discoloration and deterioration of mural paintings, respectively (Veneranda *et al*., 2017; Tescari *et al*., 2018). Residual occurrence of biogenic pigments causing aesthetic decay was also characterized (Maguregui *et al*., 2012). A role of wind direction at the regional scale has recently been reported to affect the distribution of biological patinas on differently exposed vertical surfaces of architectural elements (Traversetti *et al*., 2018).

This study first informs about and quantifies lichen diversity and abundance in a Pompeian House, addressing relationships between microenvironmental features and dominant species and giving an insight into lichen-driven deterioration issues.

2.4.1. Lichen diversity and deteriogenic potential

Archaeological sites in the Mediterranean region were recognized as hotspots of saxicolous lichen diversity: they are often characterized by a higher co-occurrence of different stone materials and heterogeneous microenvironmental conditions than the surrounding areas, thus favouring the co-occurrence of different communities and, definitely, more species (Nimis *et al*., 1987). The number of 22 species on the vertical surfaces of artificial stone materials in the 600m^2 House of the Ancient Hunt (Table 1) is analogous to that reported for the horizontal sandstone flagstones of the approx. 1000 m^2 *forum* of Baelo Claudia

(Spain), while 77 species were found on siliceous and calcareous pebbles cemented with mortar in the approx. ten times wider area of the Roman Amphitheatre of Italica (Nimis *et al*., 1998). The fact that different substrates were considered in the House (mortars, painted and plastered surfaces), but sharing the same carbonate chemistry, likely explains the relatively low number of species. Moreover, it is worth noting that the blocks of both Sarno limestone and volcanic and pyroclastic rocks in the ancient walls and modern integrations were quite uncolonized, revealing a lower bioreceptivity with respect to the surface of artificial stone materials. Accordingly, mortars and building materials were already reported as highly bioreceptive for calcicolous and rather nitrophilous species (Ariño *et al*., 1995). These latter also characterize the investigated House, with the dominant *V. macrostoma* and, subordinately, F. citrina, *F. coronata* and other species which typically occur in rather to highly eutrophicated situations (Table 1; ecological indicator values by Nimis, 2016, in S2). Within the other dominant species, for which a lower nitrophytism is reported, *D. massiliensis* often characterizes artificial stone materials, including frescoes, in both outdoor and semi-confined environments (Edwards *et al*., 1991, 1997; Seaward, 2004; Nugari *et al*., 2009). Accordingly, only this species was found on painted surfaces of the semi-confined room 6, confirming the poor bioreceptivity of semi-confined environments for most lichen species (Roccardi *et al*., 2008). *L. lobificans* gathers to the *L. nivalis* group, which was already reported on mortars in archaeological sites of Southern Spain (Ariño and Saiz-Jimenez, 2004). The dominance of few species, in terms of cover and frequency, and the local or rare occurrence of others (Fig. 2B), generally characterize lichen communities in anthropic habitats, especially at early successional stages (Nascimbene and Salvadori, 2008). In terms of maximum abundance per room, values above 80% quantified in two rooms (Fig. 2A; Table 1), on painted surfaces in particular, are similar to

cover values reported for calcicolous lichen communities in archaeological sites of Central Italy (Nimis *et al*., 1987).

Lichen communities in the Pompeian House are thus generally congruent, in terms of diversity and abundance, with those reported in other archaeological areas of the Mediterranean basin. In particular, their abundance, at least at a local scale, accounts for a remarkable potential threat for conservation. With this regard, our findings show for all the three dominant specie in the House, V. macrostoma, *D. massiliensis* and *L. lobificans*, patterns of physical or chemical interaction with the substrate which account for potential deteriogenic effects. The hyphal penetration component of *V. macrostoma*, which thoroughly affects the upper layers of the painted surfaces, including the paint layer and the upper fine part of plaster (Fig. 4A-D), promote their physical disaggregation down to 1mm in depth. Although early stages of hyphal penetration within the rock substrates is related to the intrinsic availability of discontinuities, as pores and fractures (Favero-Longo *et al*., 2009), pressures subsequently exerted by hyphal structures, during their development and because of expansion and contraction of thalli according to water availability, increase discontinuities between clasts and thus favour their detachment (Ascaso and Wierzchos, 1995; Salvadori and Casanova-Municchia, 2016). The hyphal growth around matrix fragments and along the boundaries of sub-millimetric clasts (SEM images in Fig. 4E and F, and Fig. S4), may temporarily contribute to their coherence, by adhering to and keeping them together, but is likely to have very negative consequences after the natural decay of thalli or even before, if cleaning interventions are planned without care for biodeterioration patterns (Pinna, 2017; Casanova-Municchia *et al*., 2018). Moreover, hyphal penetration may contribute to the formation of microhabitats and chemical microenvironments within the substrate and thus support the endolithic growth of other lithobiontic microorganisms

having biodeteriorative effects (de los Ríos *et al*., 2002; Sohrabi *et al*., 2017).

D. massiliensis may similarly or even more deeply penetrate the substrate, as maximum penetration depths of 20mm were recorded within carbonatic substrates (Seaward and Edwards, 1995). In comparison to *V. macrostoma*, which is not known to secrete metabolites

with acidic and chelating functions, *D. massiliensis* is recognized as a remarkable agent of biogeochemical processes at the thallus-substrate interface because of its secretion of oxalic acid (Edwards *et al*., 1997; Salvadori and Casanova-Municchia, 2016). Accordingly, *D. massiliensis* thalli contained deposits of calcium dehydrate oxalates ($CaC_2O_4 \cdot 2H_2O$), for which a physiological role in storing and releasing water to counter arid conditions was suggested (Edwards *et al*., 1997; Adamo and Violante, 2000). In terms of biodeterioration of mural paintings, the lichen released oxalic acid, responsible for acidolysis and complexolysis, dissolves calcite and metal-containing pigments, and reacts with free Ca^{2+} forming the oxalate deposits (Unković *et al*., 2017). Such process leads to pigment discoloration and long-term aesthetic disturbance because of the calcium oxalate insolubility (Adamo and Violante, 2000; Rosado *et al*., 2013), and may account for a lichen origin of oxalate deposits previously reported on Pompeii ruins (Maguregui *et al*., 2012). Beside oxalic acid, *D. massiliensis* also produces erythrin, which is sufficiently soluble in water (57 mg l^{-1}) to function as metal-chelating agent and further promote chemical deterioration (Iskandar and Syers, 1971). Similarly, *L. lobificans* release atranorin and stictic acid, potentially exerting a deteriogenic role (Ascaso and Galvan, 1976). The occurrence in ortho (adjacent) positions of these polyphenolic compounds of certain electron donors polar groups, such as -OH, -COOH and -CHO, largely determines their water solubility and metal complexing capacity (Adamo and Violante, 2000). However, *Lepraria* thalli rarely grow directly on the

lithic surface, but on soil deposits or mosses, reasonably filtering their interaction with the substrate.

Different levels of potential deteriogenic effect may be thus recognized for the three dominant species, with the threats by *D. massiliensis* > *V. macrostoma* > *L. lobificans* [quantitative estimations using the Index of Lichen Potential Biodeteriogenic Action (Gazzano *et al*., 2009) in Table S4]. Knowledge on their distribution and the understanding of conditional factors may be thus crucial to face biodeterioration hotspots, establish restoration priorities and plan preventive strategies.

2.4.2. Community variability and microenvironmental factors

The regional climate primarily influences the environmental conditions of open-air archaeological sites (Caneva and Pacini, 2008). In this context, remarkable weather fluctuations characterizing the climate of Pompeii are generally detrimental to conservation (Pérez García *et al*., 2013). In parallel, investigations on other Pompeian Houses showed that different microclimate conditions can be detected between and within the rooms of a single House (Merello *et al*., 2014), which may be crucial to drive the distribution of different lichen communities.

A microclimate sensor-based monitoring is not available for the House of the Ancient Hunt. Nevertheless, our findings show that architecture-related environmental features as material (MAT), aspect (ASP), vertical distance from the ground (HEI), horizontal distance from the nearest wall corner (DNC) and room dimension (ROD), easily evaluable and related (as proxies) to microclimatic features, are significant conditional factors to drive the distribution of different lichen communities in the House.

The high values of species turnover displayed by SDR analysis both between and within rooms (high R and R + D values in Table 2) and the plot variability within each single room (PCoA-I in Fig. S1) confirm archaeological sites as hotspots of biodiversity because of the occurrence of different microniches (Nimis *et al*., 1987). According to the multivariate analyses, HEI and ROD are main conditional factors driving lichen distribution (CCA in Fig. 4B and C) and are positively related to lichen abundance (LICH in PCoA-II in Fig. 4A). The higher the surface and the larger the room, the more the lichens: as vertical distance from the ground and room dimension are related to wind velocity and ventilation patterns (Britter and Hanna, 2003; Zhang and Chen, 2006), influencing particle life times in the air and deposition rates (De Nuntiis *et al*., 2003), parameters HEI and ROD likely influence the propagule supply necessary to start colonization. Similarly, they influence the deposition of nutrients (Britter and Hanna, 2003) which support the occurrence of species rather to highly tolerant of eutrophication (including *V. macrostoma*) at higher distance from the ground and in larger rooms (clusters *i* and *ii*, positively related with HEI and ROD). The same factors may also influence the impact of wind-driven rain as relevant bioclimatic factor driving biological covers in Pompeii (Traversetti *et al*., 2018). With this regard, high colonization on surfaces with northern and western aspects, favoured by West winds influencing wind-driven rain, is here confirmed: LICH opposed to ASP in PCoA-II (Fig. 4A) indicate a positive correlation between high lichen frequencies and NNW exposition. However, maximum cover values were observed on surfaces with SSE aspect at high distance from the ground. Increasing HEI also implies lower capillary water rise, and thus lower water availability (Hall and Hoff, 2007), which agrees with the xerophytic trait of species of clusters *i* and *ii*, and it is also congruent with their drier southern exposition (higher ASP values) and

their higher distance from wall corners, i.e. from humidity stagnation (Abuku *et al*., 2009). By contrast, species of clusters v and vi, including *L. lobificans*, grow at lower HEI, ROD, ASP and DNC values, according to their mesophyly and poor tolerance of eutrophication. Accordingly, factors regulating humidity, solar radiation and temperature were already shown to drive the distribution of lichen communities on the stone cultural heritage in the tropical area (Caneva *et al*., 2015), but also lichen distribution at the micro-scale on natural outcrops in the Mediterranean region (Giordani *et al*., 2014). Water availability, in particular, has recently been confirmed as critical factor to promote microbial colonization and improve biodeteriorative effects on the stone cultural heritage (Caneva *et al*., 2016; Liu *et al*., 2018).

The substrate material (MAT) also significantly affects the distribution of lichen communities, with the less porous, fine-grained painted and plastered surfaces revealing even higher receptivity to lichen colonization than both ancient and modern mortars (PCoA-II in Fig. 4A). The exposure of raw walls and related mortars generally characterize areas where paint and plaster layers were not recovered or conserved, implying general surface instability and conservation difficulties and thus also justifying lower lichen occurrence (Favero-Longo *et al*., 2015). In this sense, surfaces close to the ground, where *L. lobificans* and related species prevail (see Smith *et al*., 2009), seem more threatened by physical factors potentially determining instability (as capillary water rise) than by biodeterioration. By contrast, a priority focus should be rather posed on the still conserved paint and plaster layers, having their value threatened by lichen communities dominated by the highly deteriogenic *V. macrostoma* and *D. massiliensis*. A significant reduction in precipitation is expected in southern areas of Europe, associated with a lower biomass accumulation on the stone cultural heritage (Gómez-Bolea *et al*., 2012).

Nevertheless, such a new climate scenario may even imply a higher success of the lichen communities already adapted to xeric and eutrophicated conditions of Pompeian surfaces.

2.5. Conclusions

In the House of the Ancient Hunt in Pompeii, lichens display remarkable cover values and a high deteriogenic potential, due to hyphal penetration within the painted and plastered surfaces and/or the release of metabolites with acidic and chelating functions. Architecture-related microenvironmental features drive the species distribution. A higher vertical distance from the ground (HEI) and a larger room dimension (ROD) are the main conditional factors related to a higher lichen abundance and the occurrence of the potentially more deteriogenic species. A focus on microenvironmental parameters may thus support the management of biodeterioration issues, addressing restoration priorities and the definition of preventive conservation strategies.

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(following the format style of International Biodeterioration and Biodegradation)

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2.6. Supplementary materials

Fig. S1. Ordination of plots on the basis of the specific frequency data (PCoA-I). A. Each plot is indicated with the number of the room. B. Species abbreviations are reported in Table 1.

The figure shows the plot variability displayed by Principal Coordinate Analysis-I (PCoA-I), explaining 84.9% of total variance of species frequency values (details in Table S1a). The first principal coordinate (36.5%) separates V. macrostoma from other species by positive values, whereas L. lobificans is separated by negative values. D. massiliensis is discriminated by positive values of the second axis (24.4%). Plots of each room are sparcely distributed through the diagram.

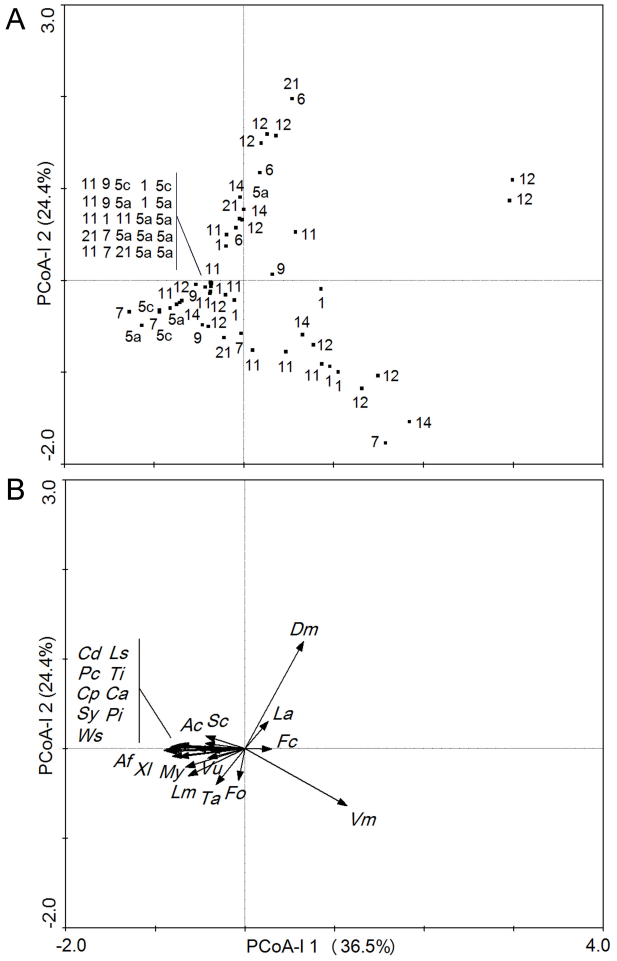
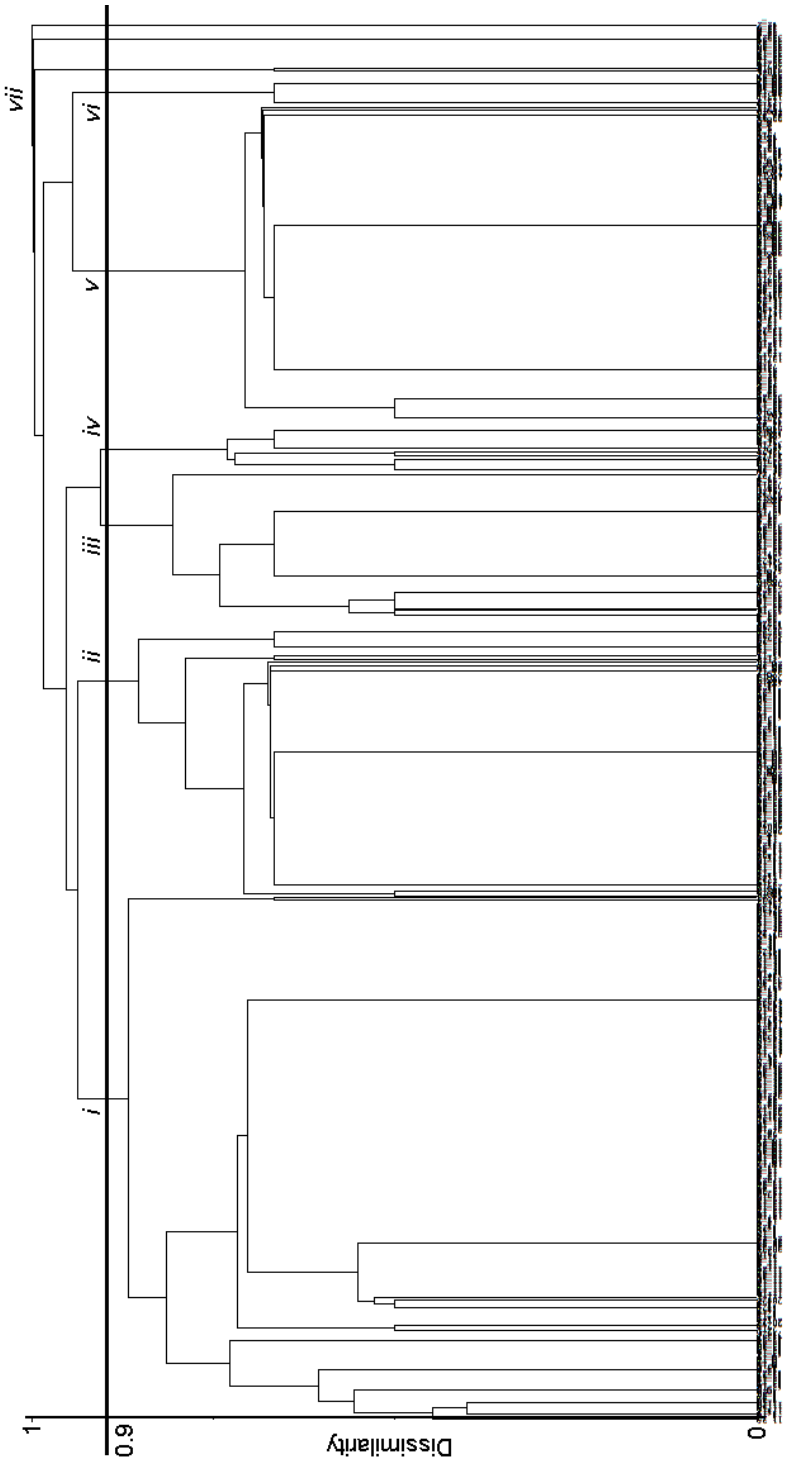


Fig. S2. Classification of quadrats on the basis of species presence/absence. Cophenetic correlation: 0.97.



Quadrats belonging to clusters i-vii (numbers in brackets distinguish different species combinations, as in Table S2).

i

(1) 11_25, (2) 6_12, 6_17, 6_7, (3) 11_14, 11_15, 11_20, 11_9, 6_10, 6_22, 6_23, 6_25, (4) 6_20, 9_8, 9_9, (5) 63_10, 63_11, 63_13, 63_16, 63_17, 63_21, 63_22, 63_6, 63_8, 63_9, 9_11, 9_12, 9_13, 9_14, 9_15, (6) 63_14, 63_15, 63_18, 63_19, 63_20, 63_23, 63_24, 63_25, 63_7, 76_24, (7) 2_1, 2_9, (8) 2_11, 2_3, 20_12, (9) 20_25, 6_1, 6_11, 6_13, 6_16, 6_2, 6_21, 6_5, 6_6, 6_8, (10) 20_24, (11) 22_1, (12) 11_1, 11_10, 11_11, 11_12, 11_13, 11_16, 11_17, 11_18, 11_19, 11_21, 11_22, 11_23, 11_24, 11_3, 11_4, 11_5, 11_6, 11_7, 11_8, 2_10, 2_2, 2_6, 20_21, 20_22, 20_23, 24_22, 24_25, 6_14, 6_15, 6_18, 6_19, 6_24, 6_3, 6_4, 6_9, (13) 11_2, 17_1, 17_10, 17_11, 17_12, 17_13, 17_14, 17_15, 17_16, 17_17, 17_18, 17_19, 17_2, 17_20, 17_21, 17_22, 17_23, 17_24, 17_25, 17_3, 17_4, 17_5, 17_6, 17_7, 17_8, 17_9, 18_12, 18_13, 18_14, 18_17, 18_18, 18_19, 18_21, 18_22, 18_23, 18_24, 18_25, 2_12, 2_14, 2_7, 2_8, 20_6, 21_10, 21_16, 21_18, 21_19, 21_21, 21_24, 21_5, 21_9, 22_11, 22_12, 22_13, 22_2, 22_3, 22_6, 22_7, 22_8, 24_10, 24_11, 24_12, 24_13, 24_14, 24_15, 24_16, 24_17, 24_18, 24_19, 24_20, 24_21, 24_23, 24_24, 24_4, 24_5, 24_7, 24_9, 45_1, 45_11, 45_12, 45_13, 45_14, 45_15, 45_18, 45_19, 45_2, 45_20, 45_23, 45_24, 45_25, 45_6, 45_7, 46_1, 46_16, 47_1, 47_10, 47_11, 47_12, 47_13, 47_19, 47_2, 47_22, 47_23, 47_3, 47_4, 47_5, 47_6, 47_7, 47_8, 53_13, 53_15, 53_20, 53_22, 53_9, 64_1, 64_10, 64_11, 64_12, 64_13, 64_14, 64_15, 64_17, 64_18, 64_19, 64_2, 64_24, 64_3, 64_4, 64_5, 64_6, 64_7, 64_8, 64_9, 7_12, 72_24, 74_11, 74_12, 74_13, 74_14, 74_15, 74_16, 74_17, 74_18, 74_19, 74_2, 74_20, 74_25, 74_3, 74_5, (14) 53_23, (15) 47_25

ii

(1) 68_7, (2) 68_10, 68_12, 68_5, (3) 23_19, 23_6, 23_9, (4) 1_13, 1_23, 16_1, 16_11, 16_12, 16_13, 16_14, 16_16, 16_17, 16_18, 16_19, 16_2, 16_21, 16_22, 16_23, 16_24, 16_25, 16_3, 16_6, 16_7, 16_8, 16_9, 18_7, 20_16, 20_17, 20_2, 20_20, 23_11, 23_12, 23_13, 23_16, 23_17, 23_18, 23_20, 23_21, 23_22, 23_23, 23_24, 23_25, 23_7, 23_8, 26_15, 26_16, 26_17, 26_18, 26_19, 26_20, 28_10, 28_13, 28_15, 28_5, 28_8, 28_9, 39_10, 39_12, 39_14, 39_15, 39_19, 39_20, 39_21, 39_22, 61_11, 61_12, 61_14, 61_16, 61_17, 61_21, 61_22, 65_11, 65_22, 65_23, 65_3, 65_4, 65_7, 65_8, 65_9, 66_10, 66_11, 66_12, 66_13, 66_14, 66_15, 66_17, 66_18, 66_19, 66_20, 66_21, 66_23, 66_25, 66_6, 66_7, 66_8, 68_15, 68_3, 78_12, 78_7, 9_7, 26_14, (5) 26_14, (6) 28_14, 28_18, 28_23, (7) 9_10, (8) 1_12, 1_7, (9) 1_11, (10) 65_12, 65_13, 65_14, 65_16, 65_17, 65_18, 65_25, (11) 52_19, 52_2, 52_22, 52_23, 52_24, 52_7, 65_19

iii

(1) 53_1, 53_2, 53_5, 53_6, 53_7, (2) 53_10, (3) 53_16, (4) 53_11, 53_12, 53_14, 53_17, 53_19, 53_24, 53_25, 53_3, 53_4, 53_8, 54_13, (5), 78_10, 78_2, 78_3, 78_4, 78_5, 78_9, (6) 54_11, 54_12, 54_17, 54_2, 54_25, 54_6, 54_7, 57_1, 57_11, 57_13, 57_14, 57_15, 57_16, 57_17, 57_18, 57_19, 57_20, 57_22, 57_23, 57_24, 57_25, 57_3, 57_4, 57_6, 57_7, 57_8, 57_9, 67_11, 67_16, 67_17, 67_21, 67_22, 68_11, 68_6, 73_11, 73_18, 74_21, 74_4, 77_1, 77_11, 77_2, 77_3, 77_6, 77_7, (7) 53_21

iv

(1) 54_14, 54_19, 54_20, (2) 54_15, 57_10, 57_12, 57_2, 57_21, (3), 63_12, (4) 54_1, 72_23, (5) 49_3, 62_13, (6) 54_10, 54_3, 54_4, 54_5, 54_9, 68_13, 68_4, 68_8, 72_21, 72_22, 76_25

v

(1) 52_13, 52_14, 52_9, (2) 49_15, 49_5, 52_10, 52_11, 52_12, 52_15, 52_16, 52_20, 52_21, 52_3, 52_4, 52_5, 52_6, 52_8, (3) 12_1, 12_6, 12_7, 12_8, 21_2, 21_3, 21_4, 7_1, 7_10, 7_7, 7_8, 7_9, (4) 1_10, 12_19, 12_2, 12_3, 12_4, 12_5, 12_9, 13_10, 13_13, 13_14, 13_15, 13_17, 13_18, 13_21, 13_3, 13_4, 13_5, 13_8, 13_9, 14_1, 14_10, 14_19, 14_2, 14_20, 14_21, 14_22, 14_24, 14_25, 14_7, 21_1, 22_17, 22_20, 22_21, 22_24, 22_25, 22_9, 23_14, 37_1, 37_11, 37_13, 37_14, 37_2, 37_3, 37_4, 37_6, 37_7, 37_8, 37_9, 38_1, 38_10, 38_11, 38_12, 38_13, 38_14, 38_17, 38_18, 38_19, 38_2, 38_20, 38_21, 38_22, 38_24, 38_25, 38_3, 38_4, 38_5, 38_6, 38_7, 38_8, 38_9, 48_1, 48_11, 48_12, 48_13, 48_15, 48_17, 48_2, 48_3, 48_4, 48_5, 48_7, 48_8,

48_9, 49_1, 49_11, 49_14, 49_2, 49_20, 49_21, 49_22, 49_4, 49_7, 49_8, 52_17, 62_10, 62_12, 62_15, 62_3, 62_4, 62_5, 62_7, 62_8, 68_2, 7_11, 7_2, 7_3, 7_4, 7_5, 7_6, 8_8, 8_9, (5) 22_16, 22_22, 22_23, (6) 49_10, 49_9, (7) 52_1

vi

(1) 13_12, 13_2, 13_7, (2) 13_1, 13_11, 13_6, 28_16, 28_17, 28_19, 28_20, 28_21, 28_22, 28_24, 28_25, 56_21, 56_24

vii

(1) 20_1, (2) 22_10, (3) 30_18, 32_1, 32_12, 32_13, 32_14, 32_16, 32_17, 32_18, 32_19, 32_20, 32_22, 32_23, 32_24, 32_25, 32_3, 32_4, 32_7, 32_8, 32_9, (4) 62_2

Fig. S3 Micro-Raman spectrum collected at the interface between Dirina massiliensis and the wallpainting in the semi-confined room 6. Bands with wavenumber at 1476, 910 and 504 cm-1 were assignable to ν(CO2) sym, ν(CC) and δ(CO2) sym vibrational modes of calcium oxalate dihydrate (Edwards et al. 2003).

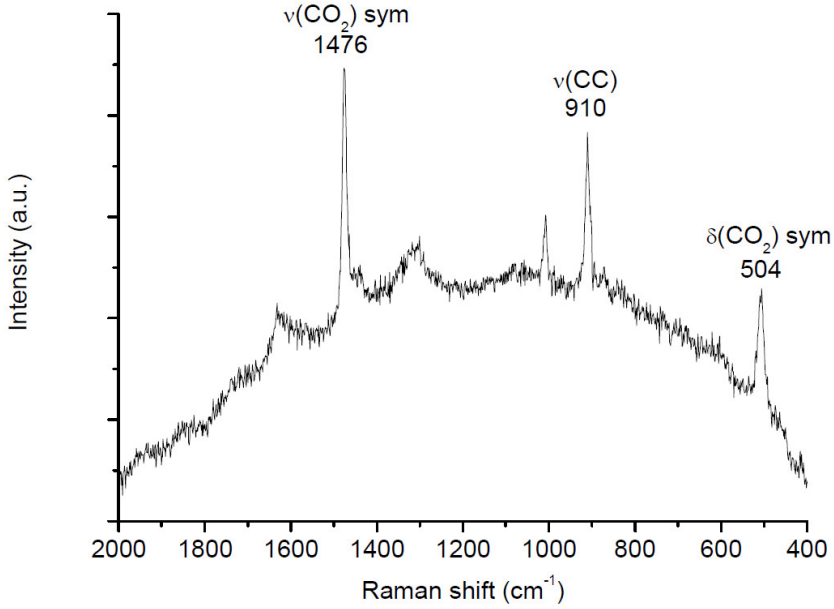
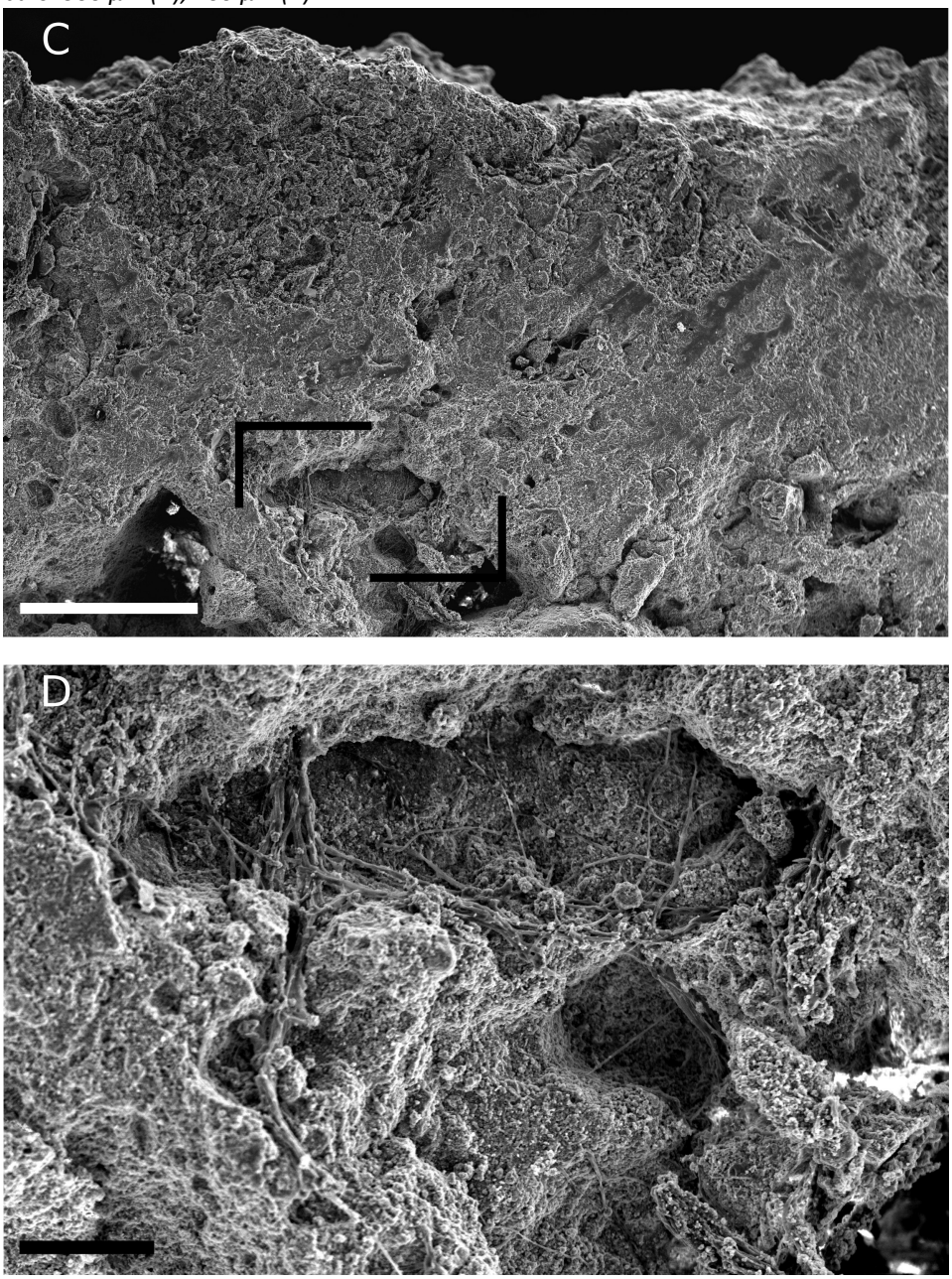


Table S1. Scores of PCoA-I (a) and PCoA-II (b).

Axes	1	2	3	4	Total variance
(a) PCoA-I					
Eigenvalues	0.365	0.244	0.155	0.085	
Cumulative variance of species data (%)	36.5	60.9	76.4	84.9	
(b) PCoA-II					
Eigenvalues	0.358	0.260	0.156	0.118	**1.000**
Cumulative variance of species data (%)	35.8	61.8	77.4	89.1	

Fig. S4. Hyphal penetration component of Verrucaria macrostoma within the paint and plaster layers (cross section observed by SEM). A (and magnification in B): hyphal growth along the boundaries of a clast (detached during the sample preparation). Scale bars: 500 µm (A), 100 µm (B).



Clusters	i										ii										iii							iv						v						vi			vii			EIV		
SC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	pH	PHO	XER	EUT
AoQ	1	3	8	3	15	10	2	3	10	1	1	35	148	1	1	1	3	3	97	1	3	1	2	1	7	8	5	1	1	6	44	1	3	5	1	2	3	4	5	6	1	2	3	111	3	2	1	3	13	1	1	19	1			
Vm	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	3-5	3-4	3-4	3-5
Dm	1	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3-5	2-3	1-2	1-2
Lm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	2-3	2-4	2-4	1
Fo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4-5	4-5	4	4-5
Fc	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	3-5	4-5	3-4	4-5
Ac	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	3-5	1-3	1-2	1-3
Vu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	4-5	3-4	3-4	1-3
Tl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4-5	3-4	2-3	1-2
La	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3-4	3	3
Sc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2	2-3	1-2
Ca	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	4-5	3-5	3-5	2-4
Ta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3-5	3-4	3-4	2-4
My	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3-5	3-5	3-5	3-4
Xl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	4-5	4	3-4	2-3
Pc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4-5	4-5	4	2-3
Ws	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	n.a.	n.a.	n.a.	n.a.
Cp	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3-5	4-5	4-5	2-3
Sy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	n.a.	n.a.	n.a.	1-3
Af	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1-3	4-5	3-4	2-3
Cd	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3-4	4	3-4	1-2
Pi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3-4	3-4	1-2
Ls	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3-4	4	3-4

Table S2. Abundance of quadrats (AoQ) with different species combinations (SC) belonging to clusters i-vii (see Fig. S1). Species abbreviations as in Table 2. Ecological indicator values according to Nimis and Martellos (2017): pH of the substrate (pH), solar irradiation (PHO), xerophytism (XER), eutrophication (EUT).

Table S3. CCA scores

Axes	1	2	3	4	Total inertia
Eigenvalues	0.440	0.258	0.194	0.100	12.131
Species-environmental correlations	0.735	0.592	0.506	0.434	
Cumulative percentage of variance					
- of species data	3.6	5.8	7.4	8.2	
- of species-environmental relation	41.3	65.5	83.7	93.0	
Monte Carlo Test	F-ratio	P-value			
Test of significance of the first canonical axis	24.114	0.0020			
Test of significance of all canonical axis	12.341	0.0020			
Inflation factor (VIF) & marginal and conditional effects	VIF	λ1	λA	F-value	P-value
Vertical distance from the ground (HEI)	1.12	0.39	0.39	21.19	0.002
Room dimension (ROD)	1.12	0.26	0.25	14.19	0.002
Aspect (ASP)	1.37	0.22	0.14	7.94	0.002
Horizontal distance from the nearest wall corner (DNC)	1.23	0.20	0.13	7.30	0.002
Dominant material (MAT)	1.07	0.17	0.16	9.18	0.002

Weighted correlation matrix	MAT	ASP	HEI	DNC	ROD
Axis 1	0.1996	-0.5229	**-0.8550**	-0.5034	-0.4945
Axis 2	0.1487	-0.0287	0.4824	-0.3208	**-0.7218**
Axis 3	**0.7589**	-0.5377	0.0892	-0.2729	0.1545
Axis 4	-0.5279	**-0.6482**	0.1607	-0.1238	-0.0576
MAT	1.000				
ASP	-0.2116	1.000			
HEI	-0.1019	0.2747	1.000		
DNC	-0.0752	0.4038	0.2682	1.000	
ROD	-0.1898	0.292	0.0566	0.1066	1.000

Table S4. Index of Lichen Potential Biodeteriogenic Activity (LPBA, Gazzano et al. 2009) calculated for the three dominant species in the House of the Ancient Hunt.

	LPBA parameters							
	a*	b	c#	d	e	f#	g	LPBA index
Species								
D. massiliensis	100	10	10	5	5	1	1	5.00
V. macrostoma	100	5	5	3	0	0.7	1	3.72
L. lobificans	100	10	1	1	3	0.1	1	2.60

** To compare the deteriogenic effect of the three species, equal cover values (parameter a) were assumed.*

 # The lowest value of parameter c was assigned to L. lobificans because its growth on the lithic substrate is commonly mediated by soil deposits and mosses. In absence of experimental observations for D. massiliensis, the maximum value was assigned to the species, according to Gazzano et al. (2009).

Preface

Meristematic microcolonial fungi are known for their ability to colonise stone surfaces in harsh conditions. Their survival is guaranteed by their defensive mechanisms of growth and reproduction, among which the production of melanin, accumulated in the walls of the fungal cells. The role of melanin in fungi is mostly related to stress tolerance, but in pathogenic and phytopathogenic microcolonial fungi it was also related to an increase of cellular turgor, assisting the penetration of fungal hyphae within vegetal tissues. A possible similar role was suggested for melanin of lithobiontic microcolonial fungi, but this theory was never confirmed.

In this chapter study, in the framework of a collaboration between the Department of Life Sciences and Systems Biology of the University of Torino and Department 4 'Materials and the Environment' of Bundesanstalt für Materialforschung und –prüfung (Berlin), a standardised *in vitro* protocol was realized to study the growth of a model microcolonial fungus, *Knufia petricola*, in different situations, and in particular with standardised substrates with different porosity and inoculated with the wildtype or with the fungus genetically modified to prevent it to produce melanins, carotenoids or both. The application of this protocol allowed not only to analyse the correlation between melanin production and hyphal penetration depth, but also to observe different growth forms produced by *K. petricola*, to describe their morphology and to hypothesise a possible ecological role for the less studied hyphal form, likely related to lack of nutrients. This information is particularly relevant in the field of cultural heritage conservation, because stone surfaces of statues and monuments are usually characterized by a scarce supply of nutrients. This growth form is

probably quite common on and within stone materials, but rarely examined because it is not easy to detect without a microscopical analysis coupled with staining of hyphal structures, which is a sampling-requiring procedure, usually not allowed on cultural heritage materials.

Chapter 3

Hyphal morphology and substrate porosity -rather than melanization- drive penetration of black fungi into carbonate substrates

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Abstract

Due to their ability to penetrate, deteriorate and discolour stone surfaces, rock-inhabiting black fungi represent a remarkable issue for cultural heritage conservation. Black microcolonial fungi (MCF) can also adapt to different environmental conditions, by converting from yeast-like morphology to a peculiar meristematic development with swollen cells (torulose hyphae, TH), to extremely thin structures (filamentous hyphae, FH). Furthermore, black MCF produce protective pigments: melanin, dark pigment particularly evident on light stone surfaces, and carotenoids. Black fungi produce melanin in critical, oligotrophic conditions as well as constitutively. Melanin function is mostly related to stress resistance and the ability of fungi to generate appressorial turgor to actively penetrate plant cells in pathogenic species. An involvement of melanins in stone surface penetration has been suggested, but not experimentally proved.

In this work, we tested the role of hyphal melanisation in penetration

mechanisms on the model black fungus *Knufia petricola* A95 in lab conditions. The wild-type and three mutants with introduced targeted mutations of polyketide-synthases (melanin production) and/or phytoene dehydrogenase (carotenoid synthesis) were inoculated on artificial carbonate pellets (pressed Carrara marble powder) of different porosity. After 5, 10, 17 and 27 weeks, hyphal penetration depth and spread were quantified on periodic acid Schiff-stained cross-sections of the pellets, collecting measurements separately for TH and FH. Droplet assays of the mutants on different media were conducted to determine the role of nutrients in the development of different fungal morphologies.

In our *in vitro* study, the hyphal penetration depth, never exceeding 200 µm, was proven to be consistent with observed penetration patterns on stone heritage carbonate substrates. Pellet porosity affected penetration patterns of TH, which developed in voids of the more porous pellets, instead than actively opening new passageways. Oppositely, the thin diameter of FH allowed their penetration independently of substrate porosity.

Instead, the long-hypothesized crucial role of melanin in black MCF hyphal penetration should be rejected. TH were developed within the pellets also by melanin deficient strains, and melanized strains showed an endolithic component of non-melanized TH. FH were non-melanized for all the strains, but deeply penetrated all pellet types, with higher penetration depth probably related to their potential exploratory (nutrient-seeking) role, while TH may be more related to a resistance to surface stress factors. In the melanin deficient strains, the absence of melanin caused an increased penetration rate of FH, hypothetically related to an earlier necessity to search for organic nutrients.

Highlights

- Morphological flexibility of rock-inhabiting MCF depends on nutrient availability.
- MCF can colonize and penetrate the substrates regardless the production of melanin.
- Substrate porosity affects the penetration pattern of different growth- forms of MCF.
- Artificial standardized marble pellets accelerate lab testing of fungal penetration.
- Conservation treatments should be preceded by lab tests on colonization patterns.

Keywords
Biodeterioration, Bioreceptivity, Black microcolonial fungi, Marble, Stone cultural heritage, Stress tolerance

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3.1. Introduction

Outdoor exposed stone cultural heritage is subject to various types of deterioration. Chemical and physical deterioration, due to the action of the atmospheric agents, is the most evident, but in the last decades the attention of the conservation scientists has been drawn towards the microorganisms which can colonize stone surfaces and cause biodeterioration [1-5]. Although stone surfaces are hostile due to the

scarcity of nutrients, sun exposure and available water, and many physical stresses, several microorganisms colonize these niches, often organized as sub-aerial biofilms (SABs)[6].

Knowledge on the relationship between microorganisms and colonized substrate drives the recognition of major threats to conservation and may address the definition of control strategies to face biodeterioration [7]. However, the multiple factors involved, including the stone, the microbes, and the surrounding environment, have often prevented a full comprehension of the mechanisms underlying biodeterioration, which was mostly examined in the complexity of field conditions.

The physico-chemical properties of the stone substrate affect its bioreceptivity, which is the aptitude of a material to be colonized by one or several groups of living organisms without necessarily undergoing any biodeterioration [8]. The mineral composition and surface properties, as roughness, control the microbial establishment [9]. Substrate porosity and texture were proved to regulate the growth of biodeteriogens within the stone, as in the case of lichens [10]. Microbial growth on monuments is mediated by several extrinsic factors, acting from a macro- (e.g. water regime, relative humidity, solar radiation, temperature, wind, atmospheric pollution) to a micro- (e.g. orientation, shading, permanent capillary humidity, etc.) scale [9,11,12].

Accordingly, protocols to test the bioreceptivity of stone materials in standardized environmental conditions were proposed and applied to evaluate the establishment and growth on mineral surfaces of stable phototrophic multi-species cultures [13-15]. However, laboratory approaches have still been poorly used to examine the microbial-substrate interaction in terms of penetration and dissolution patterns, and to explore the underlying mechanisms [10,16-19].

Microcolonial fungi (MCF) are a major component of SABs on stone surfaces in extreme environments, from Antarctica to hot deserts [20-23], and they often occur on stone cultural heritage [24,25]. Compact black microcolonies are equipped to withstand extreme environmental challenges including desiccation, UV radiation, temperature and oxidative stress as well as biocide treatments. They represent a remarkable issue for the conservation, due to their ubiquity, dark colour and the ability to penetrate and modify stone surfaces [26-29]. Black MCF are a polyphyletic group of Ascomycetes sharing morphological plasticity and functional adaptive traits which ensure their survival in extreme environments [30,31].

Since the early descriptions at the end of last century, it was recognized that rock-inhabiting black fungi can drastically change morphology depending on environmental conditions, with conversions from yeast-like cells, to a peculiar meristematic development, to extremely thin hyphae [21,32]. Their growth by isodiametric enlargement and subsequent division of cell compartments (meristeme-like), with thick and even multi-layered cell walls, gives rise to aggregated structures and swollen, 'torulose hyphae' (*sensu* De Leo *et al*. [33]). These are recognized as a prominent morphological trait of MCF, suitable to minimise the contact of the colonies with the atmosphere, and thus evaporation [30,31]. The co-development of thinner, 'filamentous hyphae' (*sensu* De Leo *et al*. [33]) and their role has been poorly discussed yet. In particular, while the differentiation of filamentous structures, called 'pseudohyphae', is well known for yeasts and has been related to nutrient limitation [34], their presence has rarely been remarked for MCF [35]. For this reason, this morphological form of growth has been poorly studied and it is still difficult to define if filamentous hyphae of MCF are proper hyphae, pseudohyphae or if it is

possible to distinguish the presence of both these growth morphologies. The production of a variety of extracellular polymeric substances (EPS), retaining water and inhibiting access of external agents, and the incrustation of the cell-wall with melanins are additional adaptive traits to increase stress tolerance [6,36,37].

The role of melanins in fungi, including in black MCF, has been related to the necessity of photoprotection, resistance to oxidation, thermoprotection, energy harvesting and metal binding [24,38,39]. Moreover, for pathogens and phytopathogens, including taxa phylogenetically close to rock-dwelling MCF, melanins have also been related to their ability to generate appressorial turgor and thus actively penetrate cells [40]. Accordingly, the involvement of these pigments in the penetration of stone surfaces has been suggested [18,19,25,33], but not experimentally proved.

Knufia petricola, a species recognized as the model MCF to study the growth and interaction of SABs with stone materials, offers the opportunity to experimentally evaluate the role of melanin in MCF penetration. This species, belonging in Chaetothyriales [41], was first isolated from a marble rock surface in Athens (Greece) and has subsequently been reported on both carbonate and silicate rock materials, including cultural heritage surfaces [28,42,43]. A melanized strain was already assayed *in vitro*, under controlled conditions, for its penetration patterns within different carbonate and silicate rock coupons, showing a high penetration rate (after four months: from few hundreds of microns to some millimetres, depending on the lithology), making it suitable for similar laboratory experiments [16]. Moreover, the development of an efficient toolkit for the genetic modification of *K. petricola*, including the deletion of genes regulating the production of protective pigments, as melanins, but also carotenoids [44-46], has

further enforced its suitability as a model organism to understand biodeterioration processes on heritage surfaces and thus address conservation and control strategies.

3.1.1. Research aim

In this work, we aimed to test the hypothesis that hyphal melanization is the adaptive trait which allows the penetration of MCF structures within the stone substrates. To work on an experimental system with a reduced number of factors involved, we developed an *in vitro* protocol including the model *K. petricola* isolate A95 [31], constant incubation conditions and standardized mineral substrates with defined porosity. In particular, the penetration of melanized and non-melanized strains of *K. petricola* was compared in terms of hyphal penetration depth and spread within carbonate pellets produced from pulverized Carrara marble. Measurements were separately performed on torulose and filamentous hyphae (*sensu* De Leo *et al*. [33]) in order to address their potentially different ecological significance. This is of particular interest for biodeterioration scientists, allowing the evaluation of the biodeterioration potential of MCF on stones of interest for conservation of cultural heritage and addressing suitable control strategies.

3.2. Materials and Methods

3.2.1. Preparation of carbonate pellets with different porosity

Carrara marble was selected to produce standardized pellets, due to its light colour and the consequent high susceptibility to the aesthetic damage caused by black fungi, and its widespread use in buildings and statuary in the past as in the present.

White marble blocks were sampled in an ancient Roman quarry near Colonnata (Carrara, Italy; UTM ED50 N4881839, E591489). The colonized and/or chemically altered volumes were removed using a diamond saw. The blocks were then manually crushed to smaller fragments and pulverized in an agate jar with agate balls, using a planetary ball mill (Planetary S100, Retsch, Germany). The powder was sifted with a 100 µm sieve and the fine powder obtained was used to produce standard circular pellets (weight: 850 mg; diameter: 12.7 mm), using a pelletizer IR-Presse 25T (Maassen GmbH, Germany). Two different pressures were applied for 60 seconds to produce pellets with different porosity: 4 tons (4T pellets; 2.70 mm thick) or 7.5 tons (7.5T pellets; 2.55 mm thick).

The porosity of the two types of pellets was evaluated with analyses on images acquired in back-scattered mode (BSE) with a scanning electron microscope JEOL JSM-IT300 (high vacuum/low vacuum 10/650 Pa; 0.3-30 kV) and the software AZtec (v.3.3, Oxford Instruments, UK). Representative pellets were cross-sectioned and semi-quantitative measurements of solids and voids were performed along vertical transects with the software WinCAM2007d (Regent's Instrument, Canada) in grey-scale mode, identifying the porosity as the black coloured areas in BSE following the approach described by Favero-Longo *et al*. [10].

4T pellets showed a rather homogeneous porosity through the whole

thickness, equivalent to approx. 50% (Fig. S1A). 7.5T pellets showed a lower average porosity of approx. 12%, which decreased to 5% in the 600 µm thick upper layer, which was in direct contact with the plunger of the pelletizer during the pellet production (Fig. S1B).

The pellets were finally sterilized in autoclave at 120°C for 20 minutes to avoid possible biological contaminations.

3.2.2. Fungal material

The model MCF *Knufia petricola* A95 was selected as fungal material [47]. The wild-type strain (wt), isolated from a marble surface in Athens (Greece; [44]), and three genetically modified strains, in which the gene of interest was replaced by a resistance cassette *via* homologous recombination, were used. To investigate the influence of the *K. petricola* pigments on the substrate penetration, a Δ*pks1* mutant with a deletion of the polyketide synthase gene within the melanin synthesis pathway, the Δ*phd1* mutant with the deletion of the phytoene desaturase for the synthesis of carotenoids and one pigmentless mutant (Δ*pks1*/Δ*phd1*) with both deletions were selected [46] (Fig. 1).

The four strains, stored at the Bundesanstalt für Materialforschung und -prüfung in cryopreservation (-80°C), were transferred in Petri dishes on nutrient-rich medium (Malt Extract Agar, MEA) to the University of Turin. Subcultures were produced and kept at the constant temperature of 20°C in a dark environment for at least 2 months to allow the growth of fungal colonies with a diameter of at least 2 cm and to proceed with inoculation on the carbonate pellets.

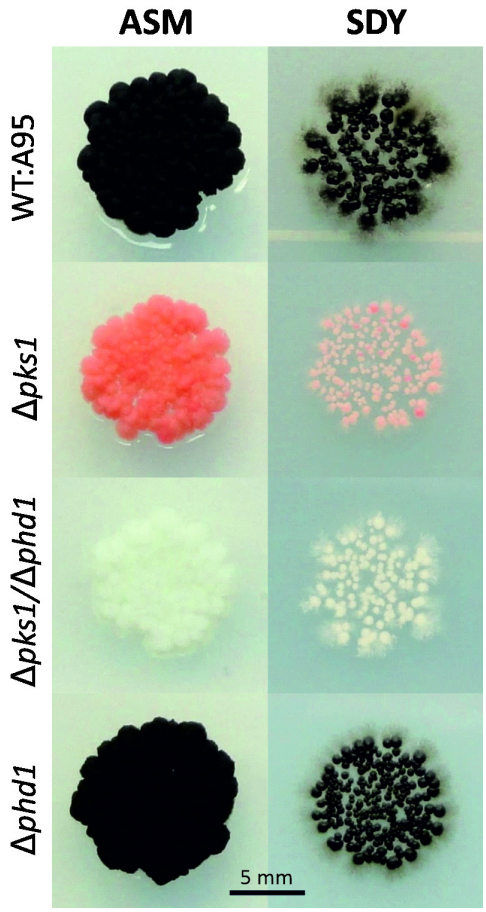

Fig. 1. Colony morphology of K. petricola *on agar with different nutrient concentration. Droplets of cell suspensions of* K. petricola *wild-type and pigment mutants were inoculated on nutrient rich medium (ASM) and on poor medium (SDY). On ASM no hyphae were observed whereas on SDY all strains formed hyphae at the border of the colonies.*

3.2.3. Fungal inoculation on agar plates for droplet assays

Cell numbers of suspensions of *K. petricola* wild-type and pigment mutants were determined with a hemocytometer and 1000 cells in a 10 µl droplet were inoculated on the nutrient-rich medium ASM [31] and the poor medium SDY (0.17% (w/v) Difco™ Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate (BD Biosciences) + 0.1% yeast

extract (Y). Plates were incubated for 14 days at 25°C in the dark until photo documentation.

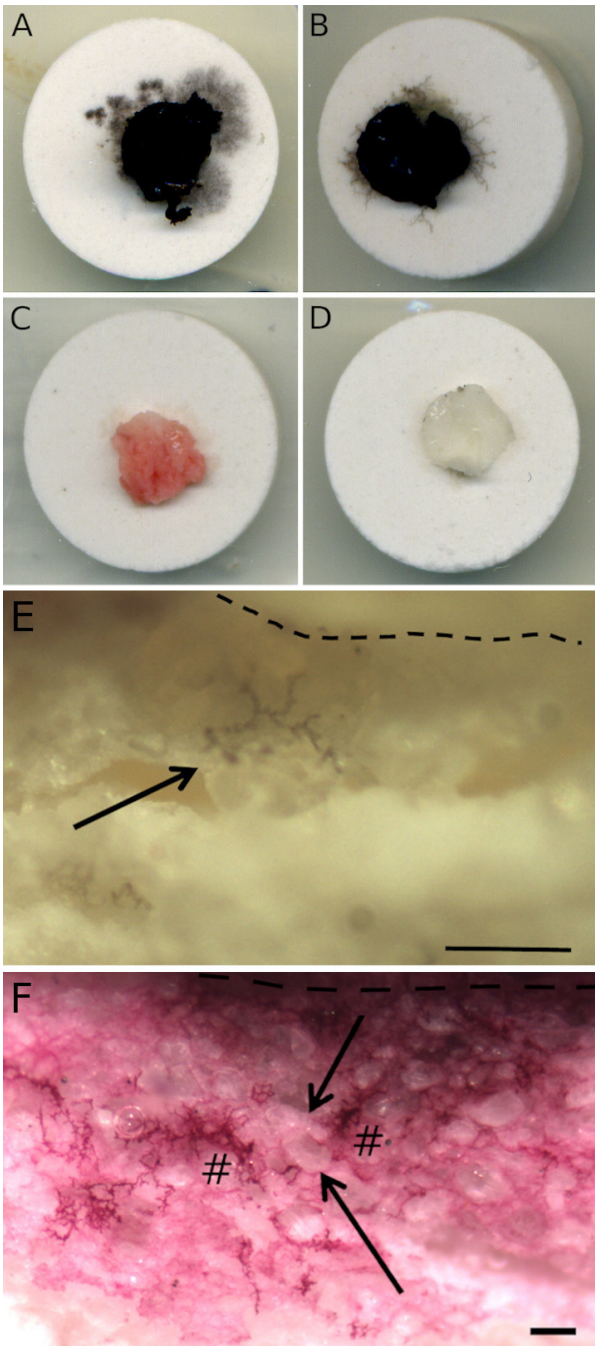

Fig. 2. Growth of K. petricola *on (A-D) and within (E-F) the marble pellets. Wildtype A95 (A); Δ*phd1 *(B); Δ*pks1 *(C); Δ*pks1/*Δ*phd1 *(D); melanized torulose hyphae developed by Δ*phd1 *within the pellet (cross-sectioned pellet before PAS-staining; E); hyphal growth within large porosities (#) and along intergranular borders (arrows) (PAS-stained, cross-sectioned pellet, F). Scales: pellet diameter =1.27 cm (A-D); bars: 100 µm (E-F).*

3.2.4. Fungal inoculation on carbonate pellets

The 4T and 7.5T pellets were placed inside Petri dishes over a thin layer of agar-water terrain (15g agar per litre), necessary to keep in position the samples and to maintain humidity. For each pellet, a circular fragment (∅ 4 mm) of mycelium was cut from a *K. petricola* subculture, cleared from MEA residues, and inoculated in the centre of the pellet surface (Fig. 2A-D). Each sample (pellet + *K. petricola* inoculum) was incubated at 20°C in a dark environment until its planned observation. For each type of pellet (4T and 7.5T), groups of five (wt) and four (Δ*pks1*, Δ*phd1*, Δ*pks1*/Δ*phd1* replicates were examined at each of the following time points: 5 weeks (t1), 10 weeks (t2), 17 weeks (t3) and 27 weeks (t4) after the inoculation. Triplicate controls (C) with no inoculation were also prepared and checked for contamination at t1, t2 and t3.

3.2.5. Microscopy observation of fungal growth

At the planned time points, each sample was preliminary observed with a stereomicroscope Olympus SZH10 to evaluate the growth of mycelium on the pellet surface. The sample was then taken from the Petri dish and the fungal inoculum was gently removed from the pellet surface using a lancet. Each pellet was embedded in a polyester resin (R44 Politex-P fast, ICR S.p.A, Italy) and then cut in two halves, using a drill with polycrystalline diamond cutters (Micromot 50/E, Proxxon, Niersdorf, Germany) and perfecting the sectioned surface with a mini hand saw. One of the two cross-sectioned pellets was stained using the periodic acid - Schiff's reagent method (PAS; [48]) to highlight hyphal growth within the mineral substrate.

Each PAS-stained cross-section was examined under reflected light

using an Olympus SZH10 stereomicroscope equipped with a digital camera to acquire images (300 ppi) of the whole sectioned surface. The images of each section were assembled (n from approx. 30 to 70, depending on the section and the time point) using GIMP 2.10.10, and a grid 100 µm wide and 20 µm high was overlapped to define vertical sectors and support the measurement of hyphal depth, respectively (n=125-130 vertical sectors, 38-42 of which covered by the inoculum; Fig. S2).

Two parameters were measured per each section: *i*. the hyphal penetration depth (HPD), obtained by averaging the maximum penetration depth per vertical sector, and *ii*. the percentage of vertical sectors (NVS) in which the hyphal penetration was observed, as informative of hyphal spread. These two parameters were separately measured for the torulose hyphae (TH) and the filamentous hyphae (FH) (*sensu* De Leo *et al*. [33]). The data collection for each section took 1-2 hrs depending on the spread of hyphal penetration.

At each time point, differences between (a) HPD and NVS of torulose and filamentous hyphae, considering strains and pellet types together; (b) HPD and NVS within 4T and 7.5T pellets, considering torulose and filamentous hyphae separately, and the four strains together; (c) HPD and NVS of the four strains, considering the pellet types and the torulose and filamentous hyphae separately, were analysed by means of ANOVA with *post-hoc* t- or Tukey's test ($P < 0.05$ as significant), using SYSTAT 10.2. Data are shown through box-plots generated with Origin 6.1.

3.3. Results

3.3.1. Fungal growth in different nutrient conditions

The four strains of *Knufia petricola* cultured on the agarized media and the carbonate pellets macroscopically differed in colour and the colony morphology. The melanized wt and Δ*phd1* were black-brown and black-grey, respectively, while Δ*pks1* and Δ*pks1*/Δ*phd1* were pink and white, respectively (Fig. 1 and Fig. 2A-D).

On nutrient-rich media ASM and MEA, the melanized strains grew on the agar surface, but also in the third dimension, becoming cerebriform with age. Δ*pks1* and Δ*pks1*/Δ*phd1* were more splattered on the agar surface and displayed, with age, folded, membrane-like structures. The colonies were built by preponderant yeast cells, with torulose hyphae also widely observed for wt > Δ*phd1*, Δ*pks1* > Δ*pks1*/Δ*phd1* (Fig. 3A). On the nutrient-poor medium SDY all strains formed highly visible filamentous hyphae at the edge of the colony (Fig. 1, Fig. 3B), less to not developed on the nutrient rich media.

The four strains exhibited mycelial growth on and within the two pellet types, but different patterns were recognizable, as detailed below.

On the pellet surfaces, the mycelial growth was remarkable at the naked eye in the case of the wt and the Δ*phd1* melanized mutant, already at the first time point and before the PAS-staining (Fig. 2A-B). Non-melanized mutants displayed a similar growth, but the mycelial development was mostly recognizable under microscopy observations. At t4, the mycelium of all the strains displayed a radial growth that extended on the pellet surface well beyond the diameter of the inoculum (up to 2 mm from its border).

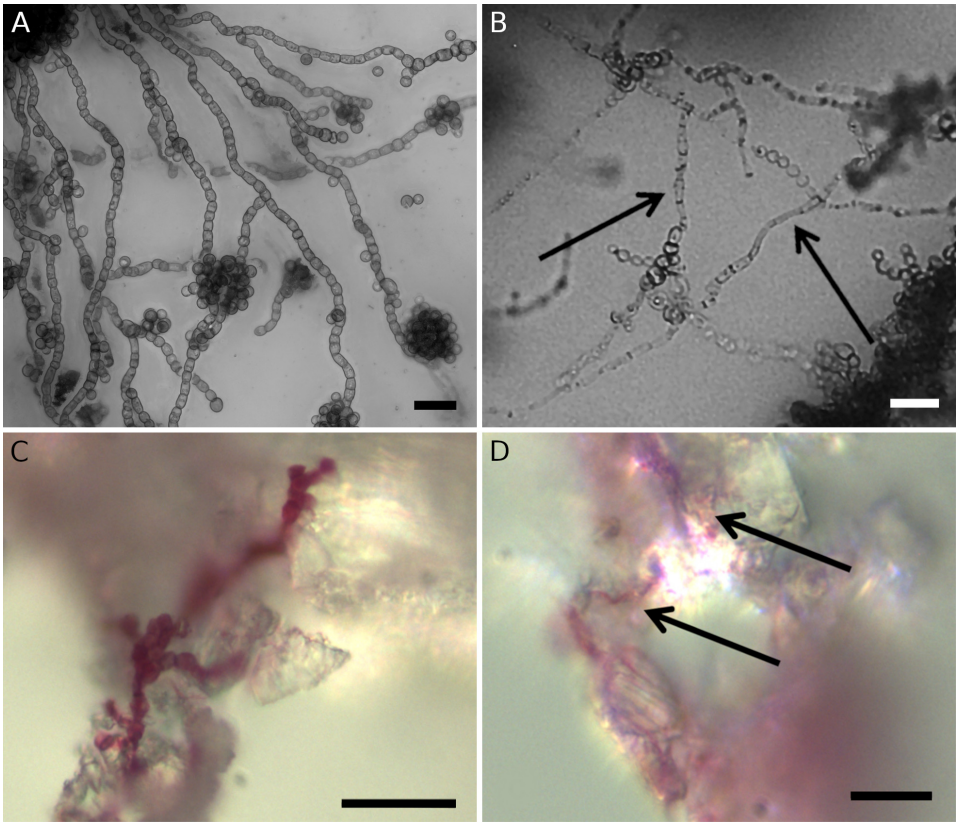

Fig. 3. Hyphal morphology on agar and marble surfaces. Torulose hyphae on agar surface, composed by swelling cells (A); filamentous hyphae (arrows) on agar surface, evidently composed by elongated, thinner cells (B); growth of torulose (C) and filamentous (D) hyphae strictly bond to the crystal surface. Scale bars: 20 µm (A), 25 µm (B,C), 10 µm (D).

Almost in every sample observed (with the exception of two samples at t1), the mycelium penetrated from the colonized surface of the pellet within its interior. Hyphal penetration started from the region beneath the inoculum and then developed also beyond the border of the inoculum. The hyphal growth was remarkably visible within large discontinuities, but it was also evident along intergranular borders, strictly bond to the crystal surfaces (Fig. 2E-F).

Both torulose and filamentous hyphae were observed on the pellet surface and within its interior. The melanized torulose hyphae were observable even before PAS staining (Fig. 2E). They were characterised by

swelling hyphae, with a diameter of 3-5 µm and meristematic growth, typical of MCF (Fig. 3A). Next to them, the co-occurrence of filamentous hyphae with a diameter < 3 µm, hyaline and elongated in the case of all strains, was only highlighted with the PAS-staining (Fig. 2F). Moreover, the PAS-staining highlighted extracellular polymeric substances (stained in pink), diffused in the volume interested by the hyphal penetration (Fig. S2).

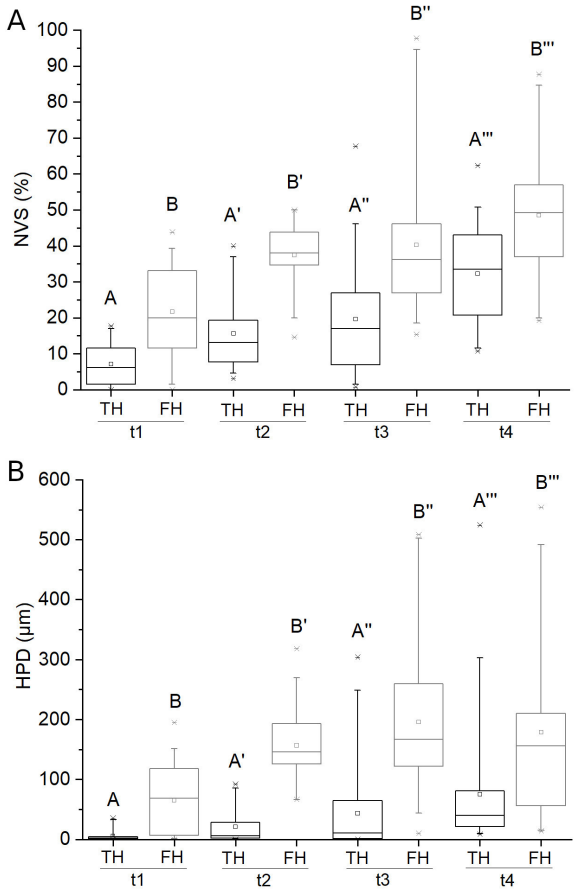

Fig. 4. Percentage of vertical sectors (NVS) in which the penetration of torulose (TH, black box plots) and filamentous (FH, grey) hyphae was observed (A), and their penetration depth (HPD) within the pellets (B), after 5 (t1), 10 (t2), 17 (t3) and 27 (t4) weeks, considering altogether the pellet types and the four strains. At each time point, box-plots marked with different letters are statistically different (ANOVA, t-test; P < 0.05).

3.3.2. Penetration patterns of torulose and filamentous hyphae

Throughout the assays, the hyphal penetration never affected more than three hundred microns from the pellet surface, and already reached its extension at t2 (approx. 20 µm growth per week from t0).

When considering altogether the four strains and the pellet types, filamentous hyphae showed higher NVS and HPD with respect to the torulose ones at all the time points (Fig. 4). Filamentous hyphae were already observable at t1 beneath the major part of the inoculum width (median NVS = 20%), and extended well beyond the inoculum border at t2 (40%). NVS of torulose hyphae more slowly, gradually increased from t1 (5%) to t4 (35%). They were mostly observed beneath the inoculum, but also far beyond (Fig. S2). The median penetration depth of torulose hyphae at t1, t2, t3 was lower than 20 µm; at t4, the median hyphal penetration reached 40 µm, with a 95th percentile at approx. 200 µm. In the case of filamentous hyphae, the median penetration was already higher than 50 µm at t1, and stabilized at approx. 200 µm at the subsequent time points (with a trend similar to NVS), with a 95th percentile above 500 µm at t4.

When considering the four strains altogether, but the pellet types separately, torulose and filamentous hyphae showed different penetration patterns. The NVS of filamentous hyphae was similar within the two pellet types until t3, and it showed a higher increase within 4T pellets only at t4 (Fig. 5A). By contrast, torulose hyphae displayed higher NVS within 4T pellets from t1 to t3, while similar values were observed for 4T and 7.5T pellets at t4 (Fig. 5B).

At all the time points, the median penetration depth of torulose hyphae was significantly higher in the 4T pellets with respect to the denser 7.5T (Fig. 5D). In particular, at t1, t2 and t3, the penetration of

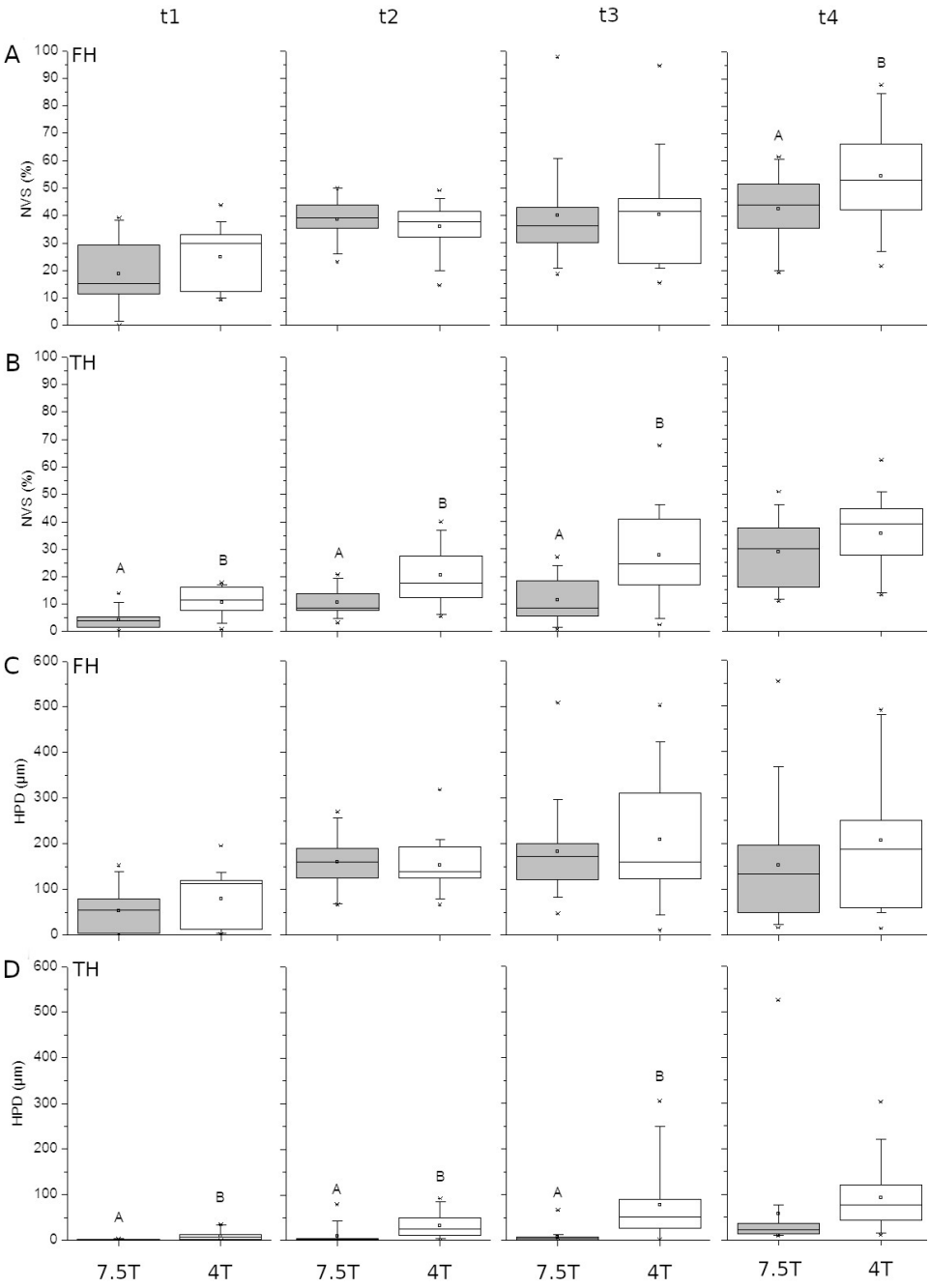

Fig. 5. Percentage of vertical sectors (NVS) in which the penetration was observed (A,B) and hyphal penetration depth (HPD; C,D) of filamentous (FH; A,C) and torulose (TH;B,D) hyphae within 7.5 T (grey box-plots) and 4 T (white box-plots) pellets, after 5 (t1), 10(t2), 17 (t3) and 27 (t4) weeks, considering altogether the four strains. At each time point, box-plots marked with different letters are statistically different (ANOVA, t-test; $P < 0.05$).

torulose hyphae within the 7.5T pellets was negligible, while it reached 10-50 µm in the case of 4T pellets. Only at t4, the median penetration depth of torulose hyphae within 7.5T pellets was 25 µm, and the 95th percentile reached 75 µm. By contrast, the penetration depth of filamentous hyphae did not show significant differences between 4T and 7.5T pellets, displaying a gradual increase of penetration values from t1 to t4 (Fig. 5C).

3.3.3. Penetration patterns of wt and mutants

Within both the pellet types, the filamentous hyphae of the four strains showed different NVS at t1, while generally similar NVS (approx. 40-50%) at the subsequent time points (Fig. 6A). At t1, in particular, wt showed lower median values than the mutant strains, within both pellet types significantly different with respect to Δ*pks1*/Δ*phd1*. At the subsequent times, only wt at t3 significantly penetrated more than the other strains within 4T pellets, while similar high values were only exceptionally observed in the other study cases (at t3, one replicate for wt within 7.5T pellets; at t4, one replicate for Δ*pks1*/Δ*phd1* within 4T pellets).

In the case of torulose hyphae, from t1 to t3, each strain showed a higher NVS within 4T than within 7.5T. However, at t1 and t2, the values of the different strains within each type of pellet were similar. At t3, wt and Δ*pks1*/Δ*phd1* displayed some higher NVS than the other mutants, but the differences disappeared at t4 (Fig. 6B).

The HPD of torulose hyphae was also higher within the 4T pellets than within the 7.5T pellets at all the time points. However, as in the case of NVS, the penetration of the four strains within each pellet type was rather identical at t1 and t2, and showed slightly higher values for

Δ*pks1*/Δ*phd1* within 4T and 7.5T pellets at t3 and t4, respectively (Fig. 6D). In the case of filamentous hyphae, HPD within 7.5T pellets was significantly higher for Δ*pks1*/Δ*phd1* than the wt and the other mutants at t1, higher for the mutants with respect to wt at t2, equal for all the strains at t3, and higher for Δ*phd1* than Δ*pks1*/Δ*phd1* at t4. Within the 4T pellets, HPD of wt was lower than the mutants at t1 (as within 7.5T at t2), equal for all the strains at t2 (as within 7.5T at t3); at t3 and t4, Δ*pks1*/Δ*phd1* was higher than the other mutants, while the wt unexpectedly showed strongly lower values at t4 with respect to t3 (Fig. 6C). In general, peculiar cases of higher or lower growth for single replicate seem to characterize the t3 and t4 time points, affecting the overall variance of the datasets.

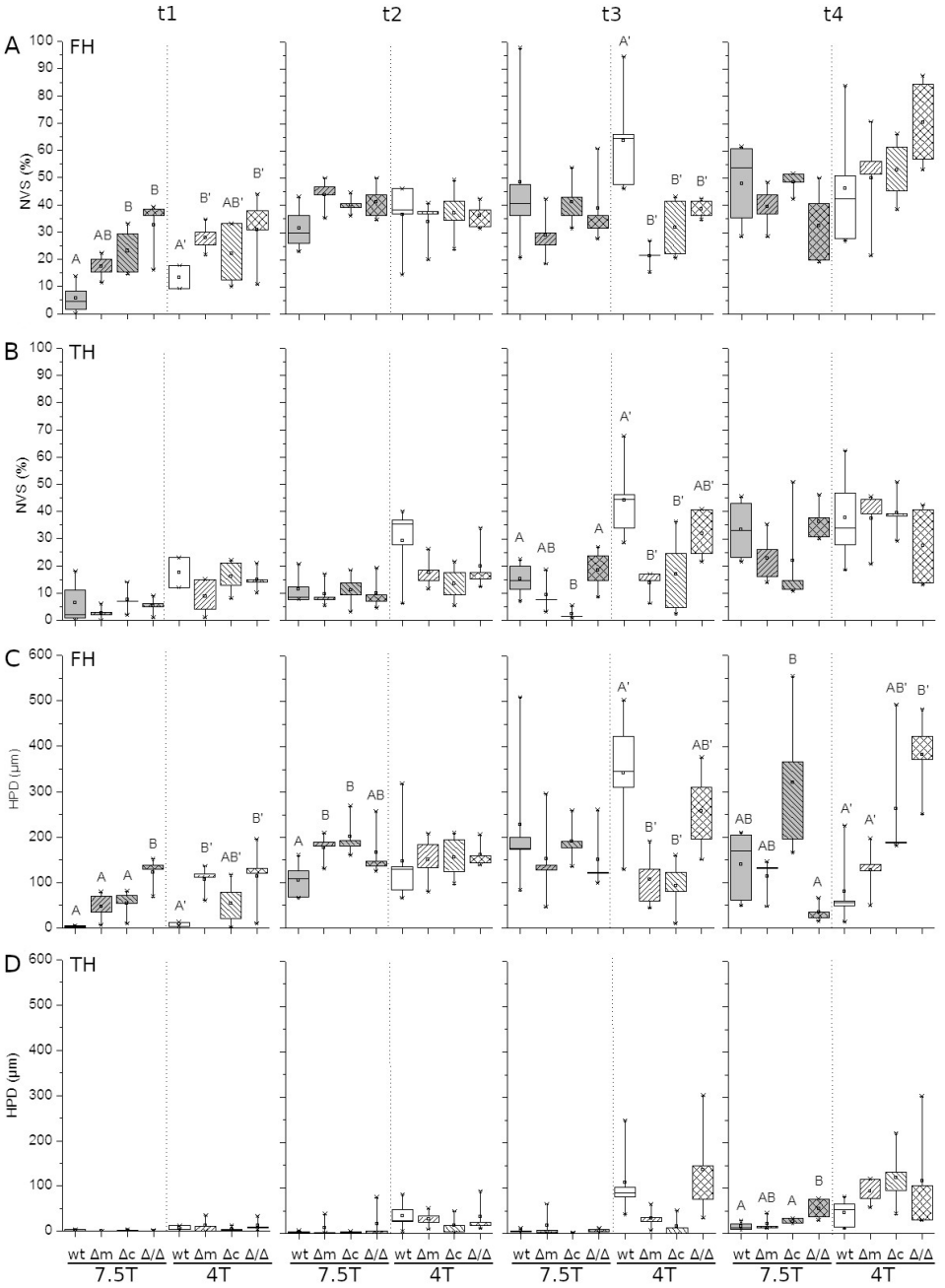

Fig. 6. Percentage of vertical sectors (NVS) in which the penetration was observed (A,B) and hyphal penetration depth (HPD; C,D) of filamentous (FH; A,C) and torulose (TH; B,D) hyphae within 7.5 T (grey box-plots) and 4 T (white box-plots) pellets, after 5 (t1), 10(t2), 17 (t3) and 27 (t4) weeks, considering separately the four strains: wt, Δpks1 *(Δm),* Δphd1 *(Δc),* Δpks1/Δphd1 *(Δ/Δ). At each time point, the box-plots of the different strains which do not share any letter are statistically different (ANOVA, Tukey's test; P < 0.05).*

3.4. Discussion

The hyphal penetration of MCF was observed within different carbonate and silicate rocks, including the stone cultural heritage, and related to their physico-chemical deterioration, but the mechanisms underlying the penetration processes have still not been fully clarified [16, 21, 33, 43]. Our investigation on the model MCF *Knufia petricola* indicates that the long-hypothesized crucial role of melanin to allow hyphal penetration by increasing cell rigidity, and thus its mechanical force [29], should be rejected. Indeed, the melanin-deficient mutant strains Δ*pks1* and Δ*pks1*/Δ*phd1* did not show significantly lower penetration with respect to the melanized wt and Δ*phd1*, both in terms of penetration depth (parameter HPD) and spread (NVS). Some higher penetration of Δ*pks1*/Δ*phd1*, lacking melanin, but also the protective presence of carotenoids, rather suggests some positive relationship between higher stress susceptibility and higher penetration. These explanations are subsequently discussed at the light of the complexity of the investigated MCF-substrate systems, including morphologically distinct hyphae [33] and pellets with different porosity, a factor significantly affecting the penetration patterns [16].

3.4.1. Hyphal morphology

The morphological plasticity of MCF is well-known and described for several species [42, 49, 50], and drastic morphological shifts following changes in microenvironmental conditions were observed, with meristematic hyphae appearing in response to temperature and desiccation stress [30, 51]. A conversion from yeast-like to meristematic growth was also considered in the description of *K. petricola*, and the

meristematic growth and penetration of torulose hyphae was associated to stone biodeterioration [32, 52]. We similarly observed yeast-like and meristematic growth patterns for the different strains cultured on nutrient-rich media (ASM, MEA), in which absence, or a strongly subordinate presence, of filamentous hyphae occurred. These latter, however, were remarkable on the nutrient-poor SDY and within the stone pellets, according to previous observations for MCF of extremely thin hyphae penetrating fissures and pores of rock substrates [21]. In particular, the co-presence of torulose and filamentous hyphae within the pellets fully mimics that recently observed by the resin-casting techniques within marble statues colonized by MCF, including *K. petricola* [33]. A higher penetration depth of the filamentous hyphae agrees with their potential explorative (nutrient-seeking) role within the bare rock material [36]. The growth of the torulose hyphae closer to the pellet surface may be related with the higher distance from the agar-water terrain on which the pellet lain within the Petri-dishes, and thus a higher desiccation stress over time [30]. This first level of observation already suggests that the torulose hyphae, usually recognized as melanized penetrating structures of MCF, may be more related to a resistance to surface stress factors than to a penetration role, which is mostly exerted by the filamentous hyphae.

3.4.2. Hyphal penetration patterns and substrate porosity

The depth of hyphal penetration observed within the pellets, in the order of few hundreds microns, was generally consistent with that observed within natural carbonate substrates, including heritage objects [27, 33]. In particular, even at t4, HPD of torulose hyphae never exceeded 200 µm, in agreement with penetrations of 100-150 µm observed for *K.*

petricola and other MCF incubated on carbonate rocks and cement slab, which are values remarkably lower than the millimetric penetration depths observed within acid lithologies [16, 53]. Beside the different ecophysiological role, the pellet porosity clearly affects the different penetration patterns of torulose and filamentous hyphae. This finding agrees with the influence of rock texture and structure on the penetration of MCF within different lithologies [16], a phenomenon mostly described until now for lichen-forming fungi [29]. Actually, the analogous HPD of filamentous hyphae within the different pellet types shows that their very thin diameters allow penetration along crystal boundaries without an influence of the different porosity. Oppositely, the development of torulose hyphae is limited in terms of HPD and NVS by the lower porosity of 7.5T with respect to 4T, and their presence seems more related to larger crevices and pores because of a sufficient void volume, in agreement with the 'penetration stage' described by Sterflinger and Krumbein [27] for MCF within marble. Similar trends observed for HPD and NVS particularly remark that torulose hyphae need available voids for their development, not only with respect to their growth in depth, but also with regard to their spread immediately below the surface. Accordingly, in the months-long monitored time, the exploitation of existing discontinuities seemed to prevail on an active opening by torulose hyphae of new passageways [54]. However, in a longer term, the chemical action of EPS [37], also highlighted within the pellets, may couple with the mechanical forces of hyphae and determine an active increase of rock discontinuities, as long supposed for MCF [6, 30].

3.4.3. Hyphal penetration patterns, melanization and stress tolerance

Multiple ecophysiological functions of melanin in fungi have been widely characterized [39]. In particular, melanin incrustation of thick-walled yeast-like cells and meristematic torulose hyphae of MCF was related with their tolerance of stress-conditions in temperature, irradiation, salt and water availability [30] and their penetration [29]. Our investigation showed the presence of torulose hyphae also in the case of melanin deficient strains, and their penetration within the substrate. Accordingly, melanin does not result the key factor driving the penetration of torulose hyphae, in agreement with early insights by Diakumaku [55] on a scarce effect of the inhibition of melanin synthesis by tricyclazole on the MCF ability to colonize marble [56]. Filamentous hyphae were always hyaline for all the strains and, nevertheless, they deeply penetrated both the pellet types. The absence of melanin prevented their observation before the staining, possibly explaining why their development and penetration was less reported than that of torulose ones in observations of MCF on cultural heritage, where their presence within stone was detected with SEM observations [33].

Although the absence of melanization did not reduce the penetration ability of *K. petricola*, it seemed to affect the penetration rate of filamentous hyphae. Higher penetration of these latter observed at t1 and t4 in the case of Δ*pks1*/Δ*phd1* may be hypothetically related to an earlier necessity to explore the substrate and find organic nutrients and/or a different stress tolerance, respectively. The degradation of melanin in the own cell walls was indeed suggested as a way of supporting the MCF metabolism in the absence of nutrients on bare rock [31]. Since this was not possible for melanin deficient strains, the explorative filamentous hyphae may have early been produced. On the other hand, the melanin deficient strains, and in particular the

Δ*pks1*/Δ*phd1* which also lacks carotenoids, should less cope with stresses [44] which may affect the colonies after the long incubation. However, the incubation at 20°C and in the dark should have mostly reduced any stress factor acting on the colonies. Just the mild conditions may have generally favoured the development of filamentous hyphae, which could be instead less adapted to tolerate the stress of the life on rocks in the environment. This is an additional, alternative hypothesis which may justify the lower records in field samples of MCF of this hyphal morphology, which may only appear as a response to certain conditions, and certainly needs further investigations to solve its ecological significance.

3.4.4. Hyphal penetration patterns and cultural heritage

The ability of MCF to cope with environmental stress factors was often related to the difficulty encountered in their removal from the cultural heritage surfaces, and associated to their resistance to biocides and other anti-microbial treatments [24, 28]. However, the inhibition of their growth by widely-used biocidal active principles, as benzalkonium chloride, was shown *in vitro* for some species [57]. Their resilience was also related to their ability to grow inside the rock substrate [58] and this hypothesis seems supported by our observation of a spread hyphal penetration. In particular, beside the evident melanized torulose hyphae, the filamentous ones may be less reached by control treatments because of the deeper penetration. Such finding remarks the importance of selecting control treatments which are efficient not only directly on the rock surface, but also against the endolithic component [59].

The recognition of porosity as crucial factor of substrate susceptibility to MCF spread suggests the potential opportunity to reduce porosity or

limit the hyphal access to porosity to contain hyphal penetration and favour a MCF control at the surface. However, MCF are able to exploit most of organic compounds as nutrients, including protective coatings and consolidants [60], and non-traditional approaches, as mineral (bio-)precipitation [61] may be more suitable against the MCF threat. Moreover, less porous lithologies should be used as new materials or for integrations where MCF are recognized as a conservation issue.

In this framework, the incubation of MCF on mineral slabs/coupons in controlled conditions is here confirmed as a suitable approach to investigate their biology and unveil their impact on cultural heritage, by limiting the influence of multiple macro- and micro environmental factors [16, 17, 27]. In particular, we highlight the suitability of standardized mineral pellets to focus on the influence of selected intrinsic properties of rock material, as porosity, on their interactions with lithobionts. Its combination with the use of mutant strains is particularly promising for future researches to further unveil the colonization mechanisms of MCF and their impact on stone durability, and to address prevention and control strategies.

3.5. Conclusive remarks

The wild-type and three mutants of *Knufia petricola* A95 developed different mycelial structures on media with different nutrient content. Morphological flexibility of black MCF involves exploratory nutrient-seeking thin filamentous hyphae combined with torulose hyphae (and microcolonies) that donate sufficient resistance to surface stress factors. This growth-form combination along with physiological protective measures (pigments, compatible solutes) makes MCF a versatile group ubiquitously present and successfully colonising porous and vulnerable

stone monuments.

On marble pellets fungal structures of all tested strains were able to penetrate into the depth (up to 200 µm) of carbonate substrates, independently of their ability to synthesise protective pigments, thus rejecting the hypothesis that hyphal melanization is the adaptive trait which allows the MCF penetration. Rather, substrate porosity significantly drives the penetration patterns of different hyphal morphologies. The fact that development of penetrative filamentous hyphae by MCF also correlates with nutrient conditions makes preliminary testing of planned conservation treatments necessary. As any external input (e.g. an impregnation or consolidating solution) is a potential source of nutrients for microbial growth, treatments may increase biodeterioration phenomena. Marble pellets that are produced by compaction possess defined chemistry and porosity and are particularly suitable for accelerated testing and selecting conservation treatments in lab bioreceptivity tests. In combination with a model rock-inhabiting black fungus and quantification analyses, this test can be established in conservation praxis when selecting restoration treatments for monument protection.

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(following the format style of Journal of Cultural Heritage)

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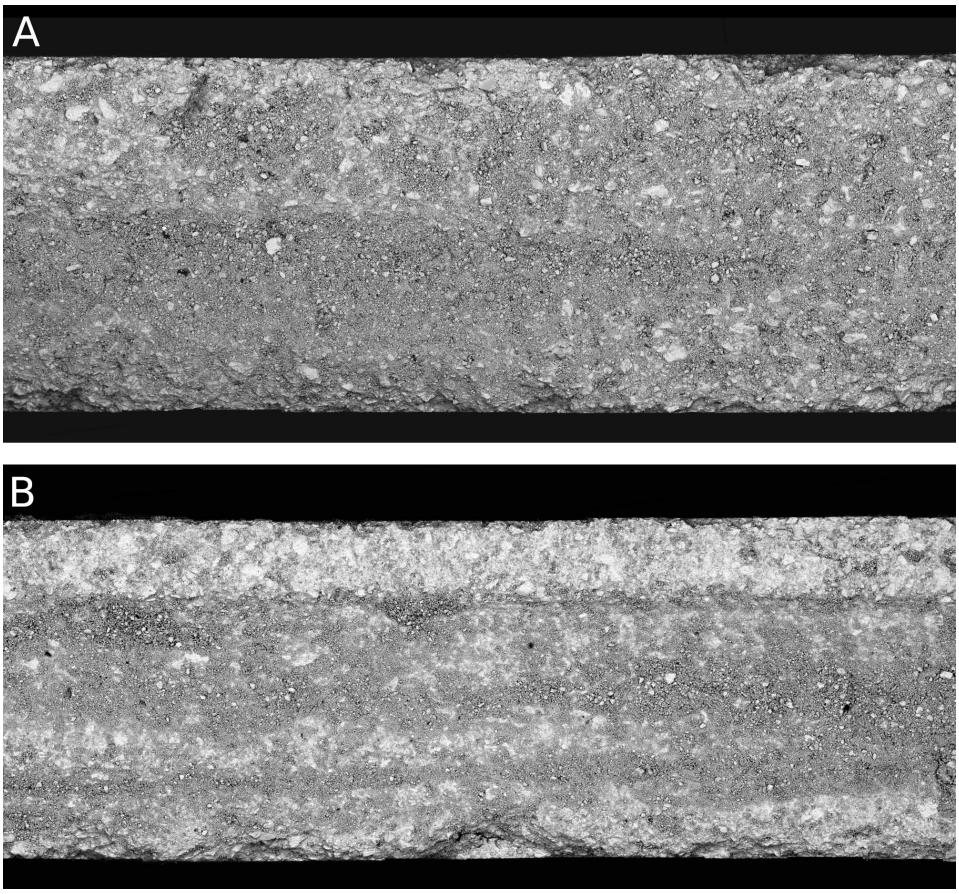
3.6. Supplementary materials

Fig. S1. Cross-sectioned carbonate pellets. 4T (thickness = 2.70 mm; A); 7.5T (2.55 mm; B).



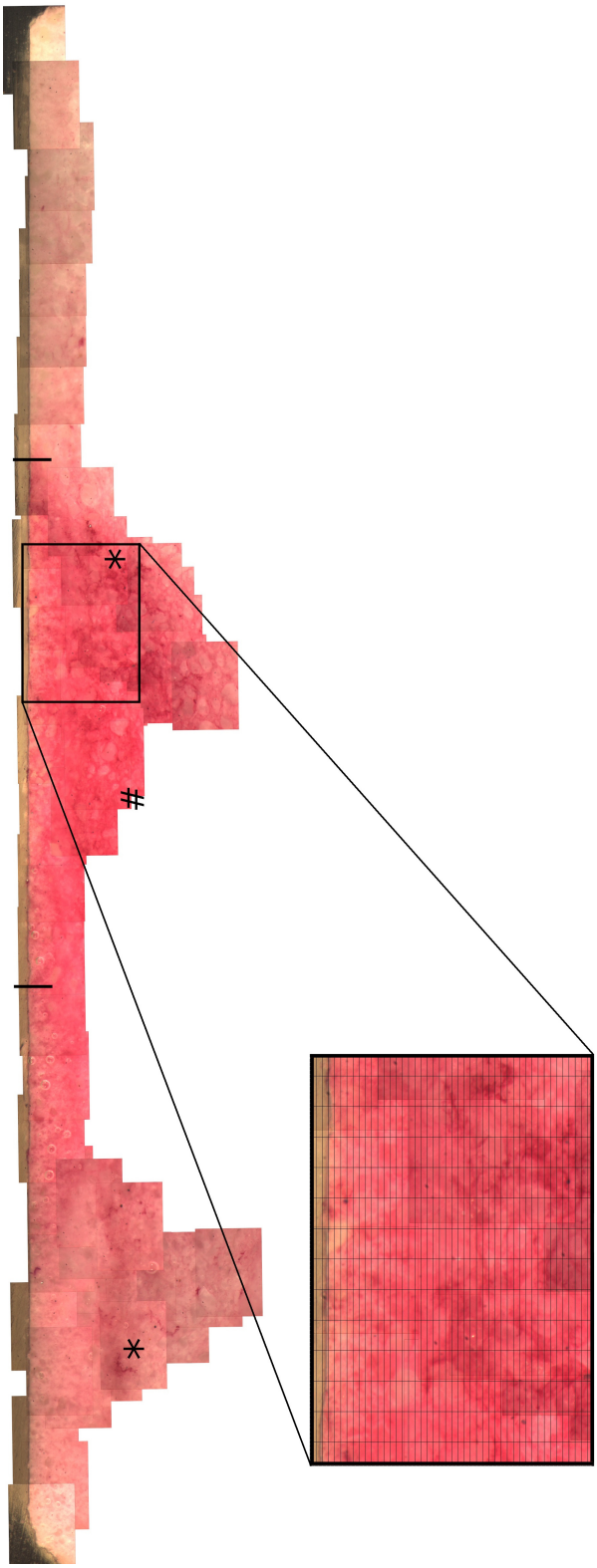


Fig. S2. Overlapping of the measuring grid (100 × 20 µm) on the assembled microscope images of a cross-sectioned, PAS-stained pellet penetrated by K. petricola*. The surface covered by the inoculum before its removal is comprised between the two brackets. Hyphae stained by PAS (violet; *) and extracellular polymeric substances (pink; #).*

Preface

Stone hardness is one of the most considered factors related to stone durability, and it has also been considered as a proxy for durability in conservation studies. As a consequence, a decrease of stone hardness caused by microbial colonization can be considered a biodeterioration effect. This correlation was previously explored in a research collaboration between Laboratory of Lichenology- Department of Life Sciences and Systems Biology (University of Torino) and Oxford Breakdown Laboratory (University of Oxford), finalised to the analysis of limestone hardness modifications beneath different lichen species, which verified that some species had a negative impact, while others neutral or even slightly positive. These observations had been paired with analyses on stone porosity variation, another proxy of stone durability, showing complex balances between biodeterioration and bioprotection effects of different species.

The conceptual idea behind that research has been further developed in this chapter, applying it on a different stone material (Cortemilia sandstone) and proposing a more rigid and standardised protocol. The method was strictly destructive, requiring to section and stain stone blocks, and for this reason the study was led on materials sampled in natural sites, close to the original quarrying area of Cortemilia sandstone used in historical buildings. Measurements of stone hardness were collected beneath three lichen species, each one characterised by different physical interactions with the substrate, but all sharing the absence of chelating metabolites, to limit the variables.

The analysis of stone hardness variation was related to hyphal penetration depth and stone mineralogical composition, analysed with

XRPD diffraction from stone portions sampled in the area beneath lichen thalli, in order to evaluate possible tracks of biomineralization processes that could have had an influence on hardness modifications. These analyses highlighted a different behaviour for lichen species, confirming that lichen removal from stone surface should keep into account the involved species and their specific effect on the material.

Chapter 4

Lichen impact on sandstone hardness is species-specific

In submission to Earth Surface Processes and Landforms

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Abstract

The balance between lichen biodeterioration and bioprotection processes on stone surfaces depends on many variables and is crucial to understanding the role of lichens in biogeomorphology and their threat to stone heritage conservation. However, stones colonized by lichens have still been mostly examined in terms of affected volumes and physico-chemical modes of interactions, overlooking the overall effects on properties related to surface durability. In this study, the impact of lichen colonization patterns on Cortemilia sandstone was examined beneath thalli of three lichen species. Rock hardness, a proxy for rock durability, was measured at different depths from the surface using an Equotip hardness tester and compared to that of freshly cut surfaces and exposed surfaces uncolonized by lichens. Mineralogical analyses were performed by X-ray powder diffraction on rock beneath lichen colonization, in comparison with unweathered rock. Equotip analyses quantified a differential, species-specific decrease in stone hardness. This variation was related to differences in hyphal penetration patterns and

calcite (re-)precipitation. In particular, in the case of the species most impacting rock hardness, X-ray diffraction patterns of calcite showed a remarkable stability of crystallographic plane (01-12), known to be enhanced in the presence of organic chelants. These results confirm that decisions on lichen removal from stone surfaces should consider species-specific behaviour. Moreover, the innovative approach of measuring stone hardness variation in association with the analysis of biomineralization processes contributes to unveil the extension of the sphere of lichen interaction within the stone substrate beyond the limit of the hyphal penetration.

Keywords
Biodeterioration, Sandstone, Lichens, Biomineralization, Stone hardness

4.1. Introduction

Lichens are self-sustaining ecosystems formed by the interaction of an exhabitant fungus (mycobiont) and an extracellular arrangement of one or more photosynthetic partners (photobiont(s)) and an indeterminate number of other microscopic organisms (Hawksworth and Grube, 2020). Saxicolous lichens represent a remarkable component of lithobiontic communities in many different environments, ranging from hot and cold deserts to tropical and temperate areas (Feuerer and Hawksworth, 2007; Wierzchoś *et al.*, 2012). The thallus of epilithic lichens develops above the surface of stones, which is penetrated by mycobiont hyphal structures only, while endolithic lichens live embedded in the mineral substrate, including the photobiont partner, with only the fruiting structures protruding (Smith *et al.*, 2009). Their colonization of

rock surfaces contributes to weathering processes, supporting pedogenesis and geomorphic transformation (Jones, 1988; Asplund and Wardle, 2016), but it is also of relevance for conservation of outdoor stone cultural heritage (Seaward, 2015). Such proven influence on shaping environment, along with their ubiquity, resulted in the last few decades in a wide interest in characterizing and modelling processes of interaction between lichens and lithic substrates (McIlroy de la Rosa *et al*., 2013).

Lichen colonization is generally associated with biodeterioration processes, which are carefully considered in the case of cultural heritage because of their negative implications for conservation (Favero-Longo and Viles, 2020). Adhesion and penetration of lichen structures, together with the release of acidic and chelating metabolites have physical and chemical impacts, exerting mechanical stress and causing dissolution and/or neoformation of minerals (Salvadori and Casanova-Municchia, 2016). On the other hand, in a small number of cases, lichen bioprotection has been documented, due to their umbrella-like action against other weathering forces (Carter and Viles, 2004; McIlroy de la Rosa *et al.*, 2014) or the sealing of rock discontinuities due to biomineralization (Lee and Parsons, 1999).

In this regard, patterns of interactions depend on colonized lithology and lichen species involved, with their different growth form, penetration, and metabolome relating to different physical and chemical deteriogenic activities (Gazzano *et al.*, 2009). Moreover, for the same species and lithologies, the balance between biodeterioration and bioprotection may change depending on (micro-)environmental conditions (Carter and Viles, 2004). However, possibly due to this wide range of variables involved and, in the case of cultural heritage, the limitations in sampling, stones colonized by lichens have still been mostly examined in terms of affected volumes and physico-chemical modes of

interactions, overlooking the overall effects on properties related to durability. Such information is needed to understand the contribution of saxicolous lichens to biogeomorphology (Viles *et al*., 2012; Viles, 2020) and their threats to heritage conservation, addressing choices of removing or preserving their colonization (Casanova-Municchia *et al.*, 2018).

In the case of limestones, lichen colonization has been associated with counterposed patterns of surface hardening and porosity increase, with their balance depending on the limestone and the species involved (Morando *et al.*, 2017). Lichen interactions with sandstones -a lithology easily targeted by microbial colonization in nature and widely used in cultural heritage- have been documented with regard to mycobiont penetration, metabolite release at the interface and changes in mineral composition (Ariño *et al.*, 1995; Bjelland *et al.*, 2002; Edwards *et al.*, 2002), but investigations have mostly neglected the consequent influence on rock physico-mechanical properties and potential divergence from abiotic and other biotic weathering (Jain *et al.*, 2009; Wang *et al.*, 2020).

The ability to measure rock hardness, which is considered to be a proxy for weathering state and durability, developed greatly in the last decade (Wilhelm *et al.*, 2016b; Kamh and Koltur, 2020). In particular, Equotip devices are widely recognized as valuable, portable instruments to measure hardness without the need for destructive sampling. Their application has been thoroughly studied and calibrated for surface deterioration on a range of materials including limestone and sandstone (Viles *et al.*, 2011; Wilhelm *et al.*, 2016a; Kovler *et al.*, 2018; Desarnaud *et al.*, 2019; Wang *et al.*, 2020). Uncertainty about possible limits to the reliability of measures taken in the proximity of edges, however, has discouraged the application of Equotip to evaluate hardness on rock cross-sections, although some studies have ruled out an edge effect,

particularly in the case of sandstone (Viles *et al.*, 2011; Desarnaud *et al.*, 2019). Such a cross-sectional approach may be particularly fruitful in evaluating the effect on hardness of lithobiontic communities including lichens, for which measurements on the colonized surfaces, “from the top”, pose the challenge of of removing the biomass without disturbing the underlying substrate and make it impossible to assay the bioweathering effects at different depths.

This research aimed to examine the impact of lichens on the physico-mechanical and mineralogical properties of sandstones related to durability. In particular, we tested the hypotheses that (a) the hardness of sandstones can be reliably quantified in proximity to block edges, (b) the hardness of sandstones beneath lichen thalli is lower than that detected without lichen colonization, and that (c) sandstone hardness at different depths beneath lichens can vary depending on their growth forms, possibly related to structural and mineralogical features of the lichen-rock interface. The investigation was carried out on blocks of Cortemilia sandstone, a lithology of interest for cultural heritage in NW Italy, colonized by epilithic, endolithic, and epi- endolithic lichens. Equotip analyses were performed at different depths on cross-sectioned blocks to evaluate variations in rock hardness. These data were associated with microscopic characterization of lichen penetration patterns and lichen-rock interface mineralogical profiles evaluated by X-ray powder diffraction, to explore possible biogenic interactions responsible for stone modification.

4.2. Materials and Methods

4.2.1. Stone and lichens

Cortemilia sandstone is quarried in southern Piedmont, Italy, and is employed extensively in local historical and modern buildings. It is a poorly sorted sandstone with very fine to medium sized grains, composed of quartz, feldspar, mica flakes, lithic grains of metamorphic rocks, and carbonate grains locally consisting of bioclasts (Gelati *et al.*, 2010). It originated in middle-late Burdigalian (early Miocene) (Ghibaudo *et al.*, 2019) from mechanical and chemical compaction, and displays a limited amount of carbonate cement.

Rock samples were collected from natural outcrops located at the top of a sunny and xeric hill with sparse trees (upstream the road SP47, Cortemilia, NW-Italy; WSG84: N 44°33'31.8" - E 8°14'45.3"), choosing or detaching blocks (n = 15) of minimal volume of 1 dm3. Selected blocks were colonized by three crustose lichen species displaying continuous thalli, namely the epilithic *Verrucaria nigrescens* Pers., the intermediate epi-endolithic *Verrucaria muralis* Ach. and the endolithic *Protoblastenia incrustans* (DC.) J. Steiner. Surfaces colonized by scarcely penetrating microbial biofilms, mostly consisting of cyanobacteria, green-algae, and subordinate black fungi, were considered as weathered controls not exposed to lichen colonization (Morando *et al.*, 2017). Lichen identification and nomenclature follow Nimis (2016).

4.2.2. Equotip hardness testing

Sandstone surface hardness was measured using a Proceq Equotip Piccolo 2 (Proceq, Switzerland) equipped with DL probe, which is very similar to D probe in terms of impact body diameter (D: 3 mm; DL: 2.78 mm) and energy (D: 11.5 Nmm; DL: 11.1 Nmm), but shows a better correlation with open porosity. Moreover, due to its geometry, DL is less prone to contamination by dust and debris which is an issue of relevance when dealing with weathered substrates (Wilhelm *et al.*, 2016a).

Hardness was measured on cross-sectioned surfaces cut with a diamond saw perpendicularly to lichen thalli, independently of any sandstone stratification, to expose the lichen-rock interface and the underlying block core. Three out of the 15 blocks (namely F, J, Z) were further cut to obtain unweathered, right-angled surfaces, to evaluate the consistency of hardness measures collected close to block edges (Fig. 1A). Cross-sectioned blocks maintained a volume > 90 cm^3 and a thickness > 5 cm, in accord with technical studies and calibrations reported for Equotip probe D and specifically carried out on sandstone materials (Corkum *et al.*, 2018; Desarnaud *et al.*, 2019). To avoid interference due to the presence of moisture contained in the rock (Desarnaud *et al.*, 2019), the blocks were left to dry for 3 weeks at room temperature to allow them to reach equilibrium. For the Equotip hardness tests, the blocks were tightly held in a bench vice, with the section face-up, to ensure surface stability and reduce as much as possible vibrations during the test.

Equotip measurements were carried out (a) at 0.2-0.3 cm from the unweathered, right-angle surface realized in the block cores (n = 3 measurements on independent cross-sections); (b) at 0.2-0.3 cm beneath

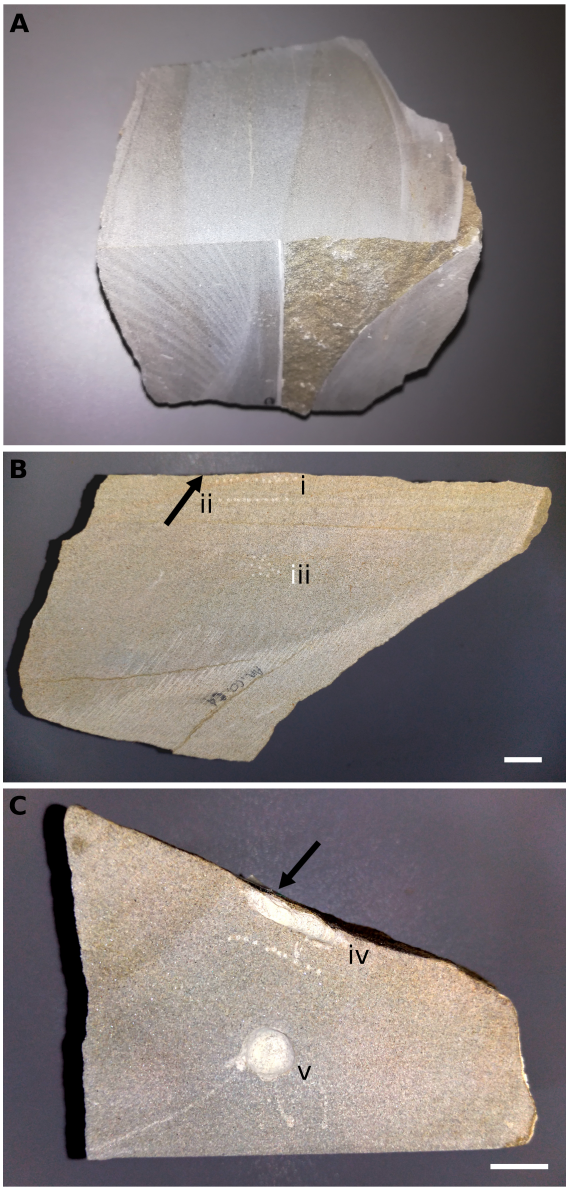

Fig. 1. Cross-sectioned Cortemilia sandstone blocks with visible impacts and sampling marks. (A) Right-angled surface obtained by cutting a block to expose its core as a model of the unweathered surface. (B) Series of Equotip measurements beneath a lichen thallus, close to the surface (i. 0.2-0.3 cm from the surface), at higher depth (ii. 1.0 cm from the surface), and in the block core (iii). (C) Powder sampling for XRPD analysis beneath lichen thallus (iv. 0.1-0.5 cm from the surface) and from the block core (v). Scale bar: 1 cm.

each thallus (*V. nigrescens*, n = 12; *V. muralis*, n = 7; *P. incrustans*, n = 8) and biofilm control (n = 12), to evaluate rock hardness as close as possible to the surface; (c) at 1 cm beneath the same thalli and biofilms, to evaluate biomodifications affecting stone hardness at greater depth; (d) at the core of each block, established as the point of the cross section as far as possible, and always at a minimum distance of 3 cm, from the

original block weathered surfaces. Each measurement consisted of a series of 11 readings, which was the maximum number of readings obtainable beneath the thalli due to their limited dimensions and thus consistently adopted for all measurements. The readings were taken in a row, parallel to unweathered edges, lichen thalli and biofilms (a-c) or sparsely in small areas of ~2,5 cm^2 (d) (Fig. 1B-C).

For each series of readings from the surface layers (a-c measurements), a relative surface hardness value (RSH, *sensu* Aye *et al.*, 2010, Kamh and Koltuk, 2020) was obtained, that is the ratio between the median value and the median value of the series of readings from the core of the same block (d measurement). The value, reported as a percentage (RSH%), represents the variation of stone hardness due to edge effects (a) and bioweathering (b-c) compared with unweathered stone hardness (d):

$$RSH_{\%} = (median_{surface\ (a\text{-}c)} / median_{\ core\ (d)}) \times 100 - 100.$$

This adjustment avoided the possibility of incorrect interpretations which might be caused by variability in hardness between different blocks.

In order to validate the method of impacting close to an edge (0.2-0.3 cm) with Equotip Piccolo 2 (probe DL) in the case of Cortemilia sandstone, variation between hardness measurements close to unweathered edges and their respective block cores was analysed by Kruskal-Wallis non-parametric test (Wilhelm *et al*. 2016b), using PAST 4.05 (Hammer *et al.*, 2001). Relative standard deviation (RSD% = SD/Average × 100, equivalent to “coefficient of variation”) for each series of measurements, excluding the two extreme values to avoid potential outliers, was also considered.

Significant differences in RSH% between the three lichen species and

the control biofilms were tested using non-parametric statistics Kruskal-Wallis and Mann-Whitney U Test, using PAST 4.05. Data are visualized as box-plots obtained using Origin(Pro), Version 2021 (OriginLab Corporation, Northampton, MA, USA).

4.2.3. Reflected light microscopy

Small fragments of the sandstone blocks from the lichen-rock interface were examined to characterise the hyphal penetration of *V. nigrescens* and *V. muralis* within the substrate. The fragments were cross-sectioned with a diamond saw, embedded in a polyester resin (R44 Politex-P fast, ICR S.p.A, Italy) and polished with silicon carbide paper (up to P1200 grit). Hyphal penetration within the substrate was stained with periodic acid – Schiff's reagent method (PAS; Whitlach and Johnson, 1974) and examined under reflected light (RLM) with an Olympus SZH10 microscope. For each section, the depth of hyphal penetration was quantified, and the thickness and density of hyphal structures were also observed. Depth data are reported as box-plots obtained using Origin(Pro), Version 2021.

4.2.4. X-ray powder diffraction

X-ray powder diffraction (XRPD) analyses investigated differences in mineralogical composition beneath *V. muralis* and *V. nigrescens*, compared with corresponding unweathered core samples. The contents of calcite and quartz, which are respectively prone and resistant minerals to (bio-)weathering induced-dissolution (Bjelland and Thorseth, 2002), were evaluated for potential relationships with sandstone hardness.

Moreover, calcite was structurally examined, focusing on the contribution of different crystallographic planes which may be informative of different crystallization and stabilization conditions (Klug and Alexander, 1974; Leoni, 2019).

Analyses were carried out on four series of samples obtained from the sections of three of the blocks used for Equotip hardness tests, selecting blocks colonized by both *V. nigrescens* and *V. muralis* and sampling in the areas of hardness measurements.

For each series, three powder samples were collected from the rock immediately beneath *V. nigrescens* and *V. muralis* thalli (0.1-0.5 cm from the surface) and from the block core (at least 3 cm from the surface), respectively. Sampling was performed using a drill/grinder (Micromot 50/E, Proxxon, Niersdorf, Germany) equipped with diamond-coated grinding bits, and the powder was manually ground in an agate mortar. The XRPD patterns were acquired with a Miniflex 600 diffractometer (Rigaku) operating at 40 kV and 15 mA, using Cu-Kα radiation (λ = 1.5406 Å), in the 2θ range of 3-70°, scan speed 2°/min with step 0.02. Qualitative and semi-quantitative analyses were performed with SmartLab Studio II v4.3 (Rigaku), using database PDF-4/Minerals 2020, to recognize main phases and peak heights referred to their different crystallographic planes.

For each XRPD pattern, the peak height (I) and the full-width at half maximum (FWHM) were calculated for the main peak (10-11) of quartz and the main (10-14) and six less intense, namely (01-12), (11-20), (11-23), (20-22), (01-18), (11-26), peaks of calcite. The I/FWHM, which can be used as an indicator of crystallinity and related to the crystallite size by the Scherrer equation (Brindley and Brown, 1980), was calculated for each listed peak.

The ratio between the I/FWHM of calcite and quartz was also calculated as follows:

$$R_{Cc/Qz} = (I\ Cc_{(10\text{-}14)}/FWHM\ Cc_{(10\text{-}14)})/(I\ Qz_{(10\text{-}11)}/FWHM\ Qz_{(10\text{-}11)}).$$

The relationship between FWHM and material hardness is a standard approach in material science (Rai *et al.*, 1999; Vashista and Paul, 2012; Fu *et al.*, 2018) and was assessed using the $R_{Cc/Qz}$ ratio for *V. nigrescens*, *V. muralis* and block core samples by a Pearson correlation analysis (PAST 4.05). For each sample series, the I/FWHM percentage variation of each calcite peak obtained for *V. nigrescens* and *V. muralis* with respect to the core was calculated and visualized using a Principal Coordinate Analysis plot (PCoA; symmetric scaling, centring samples by samples, centring species by species, performed using CANOCO 4.5, Ter Braak and Šmilauer, 2002).

4.3. Results

4.3.1. Consistency of close-to-edge Equotip impacts on Cortemilia sandstone

Three independent series of measurements from block cores and unweathered right-angled surfaces were collected to validate the method of impacting with Equotip device close to the edge (0.2-0.3 cm) in the case of Cortemilia sandstone sections.

Each block, as expected for sedimentary rocks, was characterised by a different median hardness value (e.g. variation up to 10.6% between medians). Within each block however, RSD of both core and edge series of readings was low (in the range 1.2-3.5%). Median rock hardness measured close to the edges was lower than that measured in the core, but the differences were non-significant and lower than 1.2% (Table 1).

Table 1. Stone hardness variation measured on three Cortemilia sandstone blocks under fresh, unweathered surfaces, to validate the method of impacting close to the edge (2-3 mm) of the stone with Equotip Piccolo 2 (DL probe). RSH% values represent hardness variation measured as ratio between median edge hardness and core hardness, and is always negative and inferior to 1.2% of variation. Average ±SD and RSD% are shown as informative measures of variance.

	Block F	Block J	Block Z
Core median (Leeb units)	722	683	764
Fresh edge median (Leeb units)	717	681	755
$RSH_{\%}$	-0.69%	-0.29%	-1.18%
Core average (Leeb units)	725.6	676.0	764.1
Core st.dev.	13.10	20.59	9.46
Core $RSD_{\%}$	1.8%	3.0%	1.2%
Fresh edge average (Leeb units)	723.1	683.5	752.8
Fresh edge st.dev.	13.60	24.25	10.66
Fresh edge $RSD_{\%}$	1.9%	3.5%	1.4%

4.3.2. Rock hardness variation beneath lichens

Hardness variation measured at two depths beneath lichens and the biofilm control ($RSH_{\%}$) indicated a softening of between -1.8% and –17.6% with respect to the core (Fig. 2). $RSH_{\%}$ differed significantly beneath the different lichen species. In particular, at 0.2-0.3 cm, *V. nigrescens* and *P. incrustans* showed similarly high negative $RSH_{\%}$ (approx.– 17.6%), while *V. muralis* displayed a significantly lower value (-2.4%). Biofilm control showed intermediate RSH% and a wider range of variation, but the median was closer to *V. muralis*.

At higher depth (1 cm), $RSH_{\%}$ was generally lower, and was always lower than closer to the surface (0.2-0.3 cm) for the same species, with the exception of *V. muralis* (-4.2%). In particular, $RSH_{\%}$ was particularly low for biofilm control (-1.8%), while higher for all lichen species, with more evident hardness variation for *V. nigrescens* (-10.7%) and *P. incrustans* (-8.7%).

4.3.3. Hyphal penetration and mineralogical characterization at the lichen-rock interface

PAS stained cross sections observed under reflected light microscopy showed hyphal penetration within Cortemilia sandstone beneath the structurally different *V. nigrescens* and *V. muralis* thalli, but with different patterns and depths.

V. nigrescens showed a continuous crustose thallus with typical epilithic growth, i.e. with thallus completely above stone surface including photobiont layer and reproductive structures (perithecia). The mycobiont extensively penetrated within the stone down to 1.0-1.5 mm, with hyphal network exploiting intergranular porosity and locally

organized as thick bundles (up to 50 µm), Sporadic, thin hyphae were occasionally observed at greater depths. The continuous, crustose thallus of the epi-endolithic *V. muralis* was poorly developed above the surface, showing photobiont clusters aligned at the rock surface and perithecia partially immersed in the substrate. Its mycobiont penetrated extensively down to 1.2-1.3 mm and occasionally to greater depths, as observed for *V. nigrescens*, but with less dense and thinner hyphae, only rarely organized as bundles. In a couple of cases, the early 200-300 µm beneath the algal layer appeared quite free of hyphae and seemingly more compact, while mycobiont diffusely penetrated in the millimeter below.

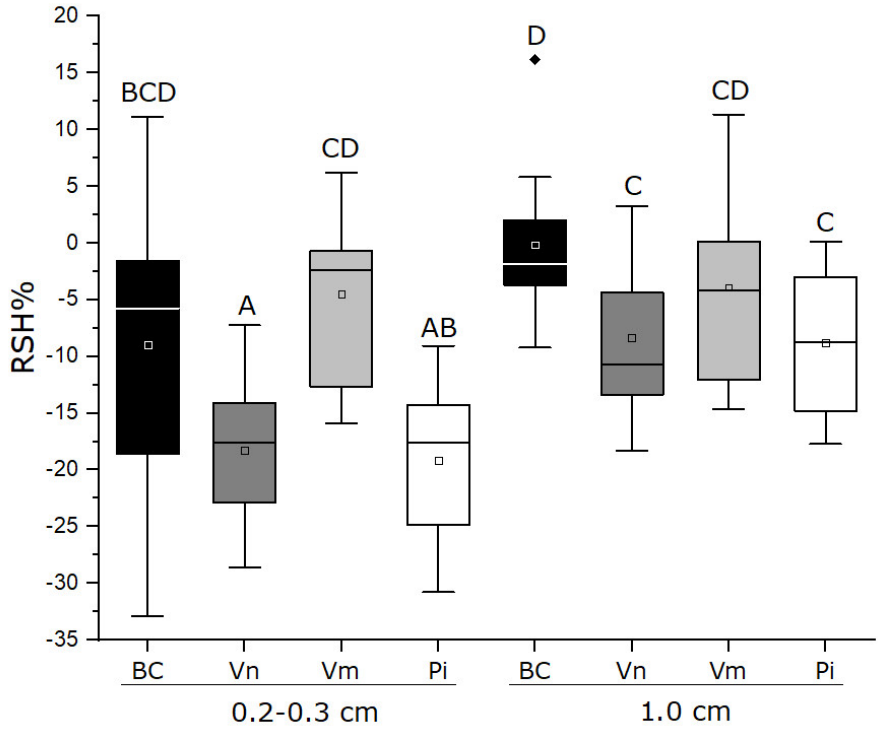

Fig. 2. Relative stone hardness variation (RSH%) beneath lichens (V. nigrescens*, Vn;* V. muralis*, Vm;* P. incrustans*, Pi) and biofilm control (BC), at different distances from the surface (0.2-0.3 cm, on the left; 1.0 cm, on the right). Box-plots which do not share at least one letter are statistically different (Kruskal-Wallis with Mann-Whitney U post-hoc test; P<0.05).*

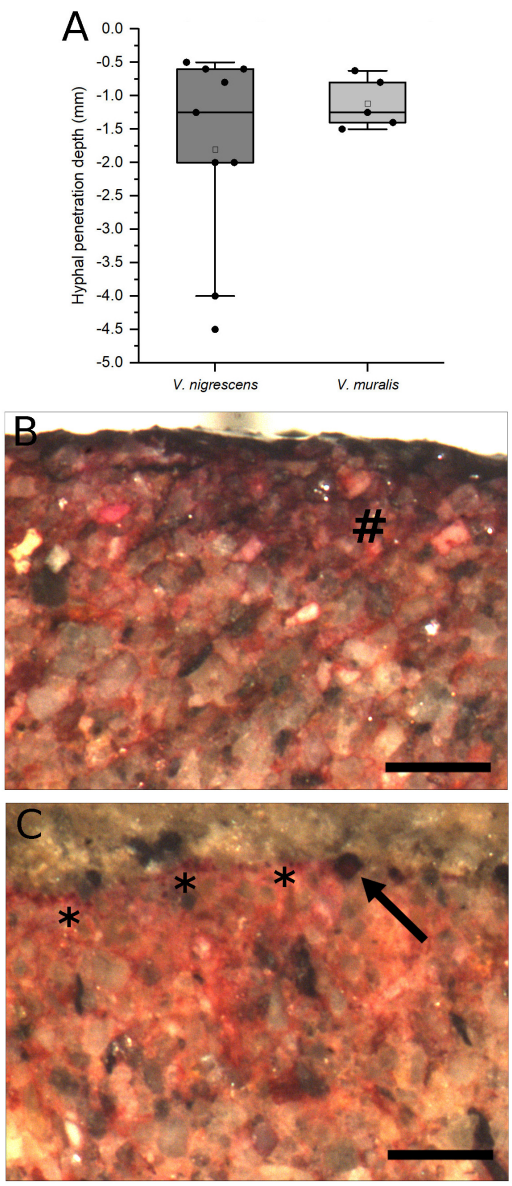

Fig. 3. Penetration depth of V. nigrescens *and* V. muralis *fungal component within stone surface. A. Difference among* V. nigrescens *and* V.muralis *penetration depth. B.* V. nigrescens *penetration patterns, with evident massive penetration of lichenic hyphae (#) red-stained by PAS coloration. C. V. muralis penetration patterns, with perithecia (arrows) partially immersed in stone. Sandstone surface is marked with dashes. Scale bars: 500 µm.*

XRPD analyses displayed similar $R_{Cc/Qz}$ values for all the samples, ranging between 0.33 and 0.72, with the exception of two core samples with $R_{Cc/Qz}$ higher than 1.75. No significant differences between *V. nigrescens* and *V. muralis* were observed. The relationship among $R_{Cc/Qz}$ and stone hardness is shown in Fig. 4A. A slight, non significant, negative correlation was observed for both the species (R = -0.8; P = 0.2).

The PCoA (Fig. 4B) extracted three components which explained

100% of total variance and ordinated samples collected beneath lichens on the basis of the percentage variation of I/FWHM ratio of each calcite peak with respect to the relative core sample. The first axis (69.9% of total variance) displayed its highest positive correlation with (01-12) crystallographic plane and with samples collected beneath *V. nigrescens* showing lowest hardness values. Oppositely, samples with highest negative correlation with (01-12) showed the highest hardness.

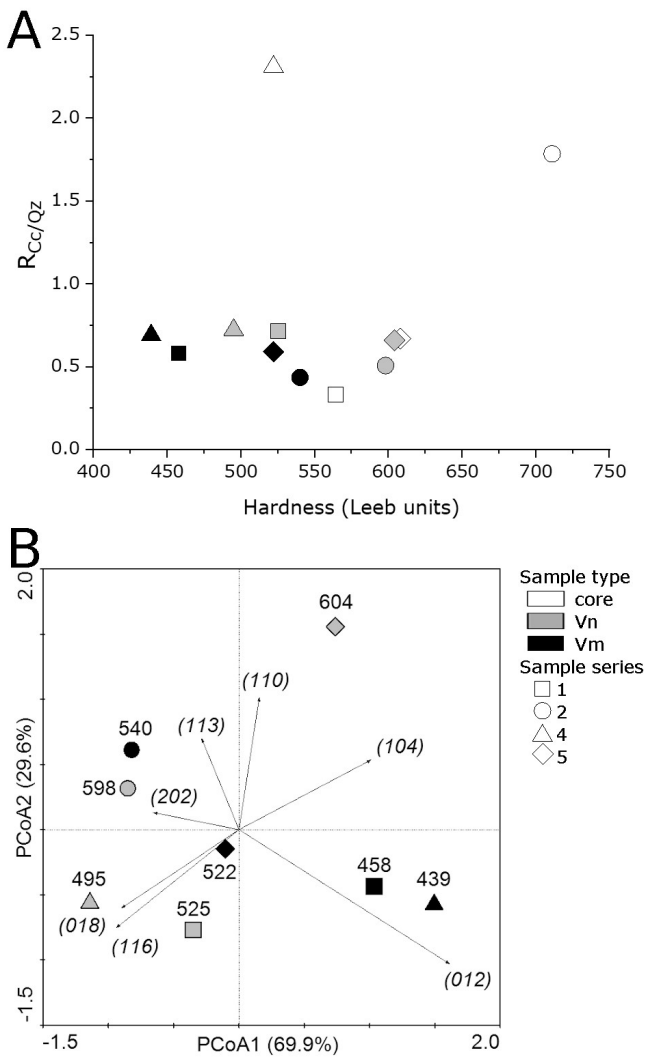

Fig. 4. XRPD analyses. A. Correlation of $R_{Cc/Qz}$ with stone hardness measured with Equotip for each sample. B. Ordination of samples on the basis of the percentage variation of I/FWHM ratio of each calcite peak referred to a crystallographic plane (PCoA vectors) with respect to the relative core sample. Median hardness (Leeb-units) is annotated for each sample.

4.4. Discussion

Analysis of lichen-rock interaction patterns may not fully clarify the balance between lichen bioweathering and bioprotection, particularly at the species specific level, which determines their biogeomorphological role (McIlroy de la Rosa *et al.*, 2013) and should contribute to decisions about whether to remove or preserve lichens on heritage surfaces (Casanova-Municchia *et al.*, 2018). Measurements of rock hardness, as a proxy of surface durability vs. erodibility (Wilhelm *et al.*, 2016b), have recently been applied to studies of sandstone weathering, encompassing lichen colonization as a general phenomenon rather than focusing on the complexity of lichen-rock interaction and species specific patterns (Kamh and Koltuk, 2020). In our investigation, we proved the efficacy of a protocol of hardness measurements at the lichen-rock interface, particularly verifying the hypothesis of (a) a non-significant influence of measuring in proximity to block edges for the examined sandstone. With such an approach, we verified the hypotheses of (b) a significant hardness variation at different depths beneath lichen thalli with respect to a biofilm control, and of (c) the differential hardness variation as a species-specific phenomenon, which cannot be directly related to epilithic or endolithic growth forms. In the following sub-sections, we discuss the adopted method to measure hardness variation with respect to most recent protocols calibrated for analyses of sandstone weathering, also taking into consideration limitations due to sampled material, variability in sandstones, and lichen thalli dimensions (*Suitability of measuring hardness along sandstone cross-sections*). Thereafter, we disentangle the patterns and species-specificity of lichen impact on a calcareous sandstone hardness (*Stone hardness variation beneath lichens*), and we address insights on the relationship between species-specific physico-chemical modes of lichen interaction, i.e. hyphal

penetration and biomineralization, and their effect on durability (*Insights on the basis of species-specific lichen impact*).

4.4.1. Suitability of measuring hardness along sandstone cross-sections

The method of measuring hardness on cross-sections of lichen colonized sandstone, including measurements close to block edges, is here proposed following previous protocols applied on fresh cut, unweathered sandstones (Desarnaud *et al.*, 2019) and on the surface of lichen colonized rocks (e.g. limestone, Morando *et al.*, 2017; sandstone, Kamh and Koltuk, 2020). Such previous protocols involving Equotip readings directly on colonized surfaces, however, reflected a cushion-like effect of the lichen biomass when thalli were not preliminarily removed. Alternatively, they required careful removal of a thallus (Morando *et al.*, 2017), which for some lithologies, as sandstone, cannot be performed without affecting the substrate. Such limitations are overcome with our implemented method of collecting measurements directly from beneath lichen thalli or other lithobionts. This confers advantages related to the possibility of obtaining innovative information of biological influence on stone properties at different depths, including the first millimetres from stone-atmosphere interface. In particular, we showed that 2-3 mm from edges obtained by sectioning blocks is a suitable distance for the Equotip probe geometry, which still ensures a reliable measurement not significantly different from (almost equivalent to) that collected at the block core. Similarly, Viles and colleagues (2011) did not observe any edge-effect for Equotip applications on sandstones, although these have been detected for other lithologies, such as granite, concrete and limestone (Coombes *et al.*, 2013), suggesting the necessity of validating the cross-section approach on each lithology of interest.

Sampling of different sandstone blocks in a natural environment implied issues related to a high variability due to different sedimentary layers, orientation, aspect, time of surface exposure, and other uncontrolled factors (e.g. Yun *et al.*, 2013). Moreover, Equotip measurements on different blocks were not always taken perpendicularly to sedimentary layers (Desarnaud *et al.*, 2019), but depending on the surface colonized by lichens and, consequently, its cross-section orientation. To balance out this variability, measurements were normalized to the core of each block, used as internal comparison. It was indeed not possible to obtain measurements of unweathered rock at the same 2-3 mm distance from the surface due to general and variable surface weathering independent of lichens.

4.4.2. Stone hardness variation beneath lichens

Hardness variation -with respect to the core- beneath lichens and the biofilm control always showed higher negative values (from -2 to -17%) than that detected beneath unweathered, right-angle edges (< -1.2%). Accordingly, the closeness to the atmosphere-rock interface and the consequent exposure to biotic and abiotic weathering agents (Gorbushina and Broughton, 2009), rather than the geometry of the surfaces related to cross sectioning, accounts for hardness variation. More remarkably, the highest hardness variation is detected beneath two of the assayed lichen species, confirming and quantifying a prominent role of lichens in rock deterioration with respect to other lithobionts (St. Clair and Seaward, 2004; Salvadori and Casanova-Municchia, 2016; Morando *et al.*, 2017).

The crustose thalli of the investigated lichen species share the absence of secreted compounds, as oxalic acid and acidic and/or

chelating secondary metabolites known for their deteriogenic activity, while they are characterized by a different structural organization (Nimis, 2016). Noteworthy, hardness variation was similar beneath the epilithic *V. nigrescens* and the endolithic *P. incrustans*, rejecting the hypothesized higher biodeterioration by endolithic lichens due to their life completely embedded in the substrate (Caneva *et al.*, 2008), but also their direct correlation with bioprotective effects (Gadd and Dyer, 2017). In detail, the hardness variation beneath these lichens is similarly high at 2-3 mm (-17.6%) and still remarkable (approx. -10%) at 1 cm, suggesting an equivalent impact for the two species on the investigated sandstone.

The detectable hardness variation at 1 cm beneath lichens is particularly remarkable with respect to the null variation driven by biofilm control with respect to the unweathered core, highlighting the deep impact of certain lichen species. Instead, at 2-3 mm, some negative hardness variation was also detected beneath the biofilm control (-6%), with higher variability possibly related to the heterogeneous biofilm composition on the different blocks. Nevertheless, a general influence of surface processes, including abiotic weathering, cannot be ruled out, as they can also significantly impact sandstone hardness (Kamh and Koltur, 2020), but their effect is more superficial than that induced by lichens.

In a very different manner, a significantly lower variation in hardness was detected beneath the epi-endolithic *V. muralis*. This was remarkably significant at 2-3 mm depth, while the variation slightly increased and reached values more similar to those of the other species at 1 cm depth. This phenomenon may suggest not a simple lower impact of *V. muralis* on stone hardness, but even some form of hardening process, which likely compensate, at least in part, stone softening caused by hyphal penetration (see below). Accordingly, hardening processes were shown for some lichen species on limestone, and in the case of Botticino

limestone the epi-endolithic *Xanthocarpia ochracea* was associated with unmodified hardness with respect to fresh rock where *V. nigrescens* determined hardness lowering (Morando *et al.*, 2017).

4.4.3. Insights on the basis of species-specific lichen impact

Different patterns of hardness variation were evaluated by comparing aspects of physico-chemical interactions of the epi-endolithic *V. muralis* with the Cortemilia sandstone with respect to the genetically related, but epilithic *V. nigrescens*.

Hyphal penetration down to millimetric depths was often reported for different sandstone lithologies (Chen *et al.*, 2000). The highest penetration values here observed for both species agreed with similar previous reports, but the massive penetration mostly affected the first millimeter only. In this regard, textural features, including porosity, have been recognized as first determinants of the rock susceptibility to colonization and, particularly, to hyphal penetration (Cámara *et al.*, 2008). The absence of significant interspecific variation in hyphal penetration between *V. nigrescens* and *V. muralis* likely reflects the availability of passageways in the first upper millimeter, although it is always difficult to ascertain whether hyphae exploit existing discontinuities and actively contribute to produce new fissures (Ascaso and Wierzchoś, 1995). Surface layers of sandstones beneath lichens displayed a porosity due to dissolution of calcite and other poorly stable minerals (Bjelland and Thorseth, 2002). Accordingly, XRPD analyses showed strongly higher $R_{Cc/Qz}$ for two core samples with respect to the related volumes beneath both the *Verrucaria* species, indicating some calcite dissolution. The absence of surfaces free of lichen or microbial colonization, however, prevented the possibility of verifying if such

pattern is directly related to biological activity or is related to abiotic weathering factors, pre-dating lichen colonization (Turkington and Paradise, 2005). For the other two sample series, the low $R_{Cc/Qz}$ characterized for core samples, equal to that obtained for volumes beneath lichens, likely reflects an initial lower content of calcite, whose amount is known to vary in the Cortemilia sandstone (Gnaccolini and Rossi, 1994). In all cases, however, $R_{Cc/Qz}$ neither showed significant correlation with stone hardness nor explained different hardness beneath the two lichen species, excluding that calcite dissolution alone accounts for the hardness variation with respect to the core. Moreover, a partial dissolution, rather than the complete absence of calcite reported by Bjelland and Thorseth (2002), was detected through the whole set of samples collected in the 5 mm deep layer beneath the lichen thalli. Although a bioprotective umbrella-effect of lichen thalli on calcite-rich lithologies was experimentally demonstrated (McIlroy de la Rosa *et al.*, 2014), such heterogeneity explains that many other factors, dealing with the overall history of stone surfaces, may account for their currently observable physico-chemical properties and the consequent conservation condition.

It is worth noting that maximum values of massive penetration were observed for *V. nigrescens,* which also displayed a denser hyphal presence in the penetrated layer, with hyphal bundles and thicker hyphal network. Such penetration patterns are consistent with pervasive penetration observed for *V. nigrescens* within limestone (Favero-Longo *et al.*, 2009), which was related to its high negative impact on limestone hardness (Morando *et al.*, 2017). Anyway, these slight differences between *V. nigrescens* and *V. muralis* are unlikely to explain hardness variations beneath the two species, mostly because hyphal penetration only rarely affected the stone deeper than 2 mm, while close-to-surface

hardness measurements were collected at 2-3 mm from the surface. The detected penetration patterns seem thus to reject the hypothesis of an exclusive role of the mechanical action of hyphae in determining stone hardness modification.

Higher heating of rock surfaces beneath *V. nigrescens* with respect to white lichen thalli was also indicated as responsible for the high stress rate induced on rock stability (Carter and Viles, 2004). However, XRPD analyses added further insights on the biogeochemical side of *V. nigrescens*-sandstone interaction. The absence of oxalates confirmed that oxalic acid -recognized as a factor responsible for bioweathering by other lichen species on sandstone (Edwards *et al.*, 2002)- is not the main driver of the biodeterioration induced by Verrucariales, as already ascertained for endolithic species of the order (Pinna *et al.*, 1998) with the exception of *Verrucaria rubrocincta* (Bungartz *et al.*, 2004). The different peaks of calcite are instead informative on the stability of different crystallographic planes, which are known to be differently enhanced in presence of organic substance (Klug and Alexander, 1974; Leoni, 2019). In particular, calcite form (01-12) is stabilized by organic chelants (Pastero *et al*., 2003). Accordingly, the correlation between the lowest hardness values observed beneath *V. nigrescens* and (01-12) may be explained by exposure of the assayed stone volumes to organic chelants, indicating that lichen impact on the stone extended beyond the hyphal penetrated volume through metabolite release. Although the production of lichen secondary metabolites is not a trait of Verrucariales, the release of chelating compounds was already observed for endolithic species (Favero-Longo e*t al.*, 2011) and may be a more widely shared feature, which does not leave prominent traces as oxalates. In this sense, we cannot exclude the possibility that *V. muralis* also releases metabolites affecting the rock stability, but the phenomenon is not reflected in the observed calcite crystallization and, if it exists, may be

more limited in line with the poorly developed biomass, above and within the substrate.

Dissolution and re-precipitation of calcite associated with lichen colonization was already characterized for endolithic species and associated with a respiration-induced acidification pathway (Weber *et al.*, 2011). Lichen biomineralization of micrite was recognized as a bioprotection factor, counterbalancing the deterioration induced by hyphal penetration (Bungartz *et al.*, 2004). The same presence of organic matter may be the cause of hardening of upper rock layers, as already demonstrated for microbial biofilms (Slavík *et al.*, 2017), but still poorly explored for lichens (Morando *et al.*, 2017). Our analyses did not allow us to exclude the possibility that the low impact of *V. muralis* on rock hardness may result from similar re-precipitation and hardening processes, as suggested by the observation of a layer appearing more compact just beneath the algal clusters, whose investigation will be the object of a subsequent contribution.

4.5. Conclusive remarks

This investigation showed that each lichen species may have a different impact on physico-mechanical properties of sandstones, as measured by surface hardness, a proxy for durability. Accordingly, a reliable evaluation of biogeomorphological processes affecting sandstone cannot generalize lichen contributions as biodeteriorative or bioprotective, or univocally associate a certain effect with the epilithic and endolithic growth forms, but rather needs to disentangle and summarize the heterogeneous contributions of different species. Such species-specific patterns are known -and here confirmed- to depend on the balance between several mechanisms of physico-mechanical and chemical impact which positively or negatively impact substrate durability. However, chemical processes may not always leave prominent evidence of their occurrence and extension at depth, as in the case of the investigated species which do not produce oxalate deposits. Our analyses suggested that microscopy observations may be integrated with mineralogical investigations, to unveil the extension of the sphere of lichen interaction within the rock substrate beyond the limit of hyphal penetration, by highlighting deeper traces of biomineralization processes. Such findings are also of relevance in the field of cultural heritage conservation, indicating that decisions on the preservation or removal of lichens, as agents of biodeterioration or bioprotection, cannot be generalized, but should carefully consider the behaviour of each species, at least focusing on dominant ones.

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(following the format style of Earth Surface Processes and Landforms)

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Preface

Removal of biological patinas from stone surfaces is a procedure that should be considered on a case-by-case basis, but in practical terms it is often considered as a standard approach to preserve stone materials and to allow their correct interpretation. Patina removal should determine on the material an impact as minimal as possible, and should be preceded by biocidal treatments. However, not all the compounds display the same efficacy on every type of biological patina, and sometimes even different species of the same group of organisms (e.g. different lichen species) can show different resistance.

The compound efficacy depends on a variety of factors. For example, the mineralogical composition and microstructural features of the colonized stone, which can also be related to its pre-existing deterioration condition, can influence the retention of biocide after its application, possibly releasing it in subsequent time and delaying the re-colonization. Another factor is the (micro-) climatic / environmental condition, which has an influence on the hydration and physiological activation of lithobionts, and might be responsible for a different biocides absorption. In this sense, even the method of biocide application and subsequent steps of the biocidal treatments can be relevant to determine the efficacy.

Restorers approaches do not usually keep into consideration all of the possible variables influencing devitalization efficacy, due to practical reasons, but also to an absence of coherent and defined data regarding this topic. As a remarkable consequence, non-calibrated procedures may lead to ineffective treatments, which often are followed by rapid re-colonization which can be even more damaging than the original one.

In the following paper, the problem of biocide efficacy is approached

with an *in situ* study, comparing the effects of biocidal compounds on foliose lichens *Xanthoparmelia tinctina*, under different conditions of thallus hydration and application methods. Biocidal efficacy was assessed with vitality measurements collected on lichen thalli before and after the treatments by monitoring chlorophyll *a* fluorescence. This research was developed in the framework of the activities of the Working Group for Cultural Heritage of the Italian Lichenological Society.

Chapter 5

The application protocol impacts the effectiveness of biocides against lichens

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Abstract

This work analysed the influence of different application protocols on the efficacy of two biocides against the foliose lichen *Xanthoparmelia tinctina* on the sandstones of the Roman Archaeological site of Luni (Italy). The hypotheses that (a) biocide application tools (brush vs. poultice), (b) pre-treatment hydration, and (c) post-treatment washing may affect devitalization success were verified by monitoring chlorophyll

a fluorescence of thalli, both *in situ* and in laboratory conditions. The hypothesis that (d) stone substrate may act as reservoir for later biocide release under repeated cycles of wetting and drying was also assayed. Analyses confirmed the importance of the application tool, with cellulose poultice being more effective than brush. Hydration influenced the biocide absorption by thalli. Moreover it modulated the metabolic activity and susceptibility to the available toxic compound, hindering lichens from entering a dormant state to tolerate stress. Depending on the preparation solvent (water vs. white spirit), the biocide application benefited from pre-treatment hydration and/or a post-treatment washing. Lastly, we showed that different sandstones variously adsorb the biocides and potentially contribute as a reservoir for their long-term release at low concentrations during successive hydration events.

Keywords

Benzalkonium chloride, Chlorophyll *a* fluorescence, Lichen, Thallus hydration, Stone conservation

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5.1. Introduction

The growth of lithobiontic (micro-)organisms widely affects the aesthetic and threatens the durability of heritage surfaces (Caneva *et al*., 2008; Negi and Sarethy, 2019). In particular, lichens are primary agents of stone biodeterioration. Their metabolites induce mineral leaching and biomineralization, and their hyphal penetration promotes disaggregation processes (Adamo and Violante, 2000; Favero-Longo *et al*., 2005; Seaward, 2015). Despite some bioprotective effects are recognized for certain species on certain lithologies (Salvadori and Casanova-Municchia, 2016), the removal of lichens is generally considered pivotal to preserve heritage surfaces and is standard practice in conservation and restoration plans (Pinna, 2017).

Physical methods for the control of lithobionts (e.g., electromagnetic wavelengths, laser and temperature shifts) have attracted recent research interests and showed promising results (e.g. Tretiach *et al*., 2012; Mascalchi *et al*., 2015; Sanz *et al*., 2015; Rivas *et al*., 2018). Nevertheless, their optimization and practical applicability at the scale of monumental surfaces is still pending (Pozo-Antonio *et al*., 2019; Sanmartín *et al*., 2019). Accordingly, interventions including devitalization of thalli by biocide application, followed by their removal by mechanical methods, are still commonly used by restorers (Kakakhel *et al*., 2019). Killing lichens prior to their brushing or scraping from the stone surfaces is recognized as a crucial need to prevent the persistence of viable thalline fragments within rock fissures and the dispersal of propagules, which may promote rapid recolonization processes (Pinna, 2017). However, the effectiveness of biocidal treatments against lichens is not generalizable, and unsuccessful applications are widely documented in terms of poor devitalization results as well as of an undesired boosting of more resistant and aggressive species (Seaward,

2015). It has been demonstrated that the effectiveness of biocidal products is species-and site-specific and it is strongly influenced by the application tools adopted (Favero-Longo *et al*., 2017). *In situ* preliminary assays are thus necessary to evaluate the site- and species-specific devitalization power of biocidal products and application tools, before their wide scale use in restoration interventions (Ascaso *et al*., 2002; de los Ríos *et al*., 2012; Favero-Longo *et al*., 2017; Pinna, 2017). Certain practical steps of biocide application, which may affect their effectiveness, are similarly worthy of investigation to validate protocols ensuring the devitalization success.

Different substrate lithology and (micro)climatic conditions are site-related factors which may alter the effects of biocide applications (Caneva *et al*., 2008; Salvadori and Charola, 2011). In strict relation to microenvironmental variation, the susceptibility of lichens to stress factors depends on their hydration state. They are stress-tolerant when dry, while highly sensitive when hydrated (even partially) and thus metabolically active (Tretiach *et al*., 2012). However, the choice of applying biocides on previously hydrated or dry thalli is still a controversial issue. Two contrasting hypotheses have been formulated, postulating that the pre-hydration of lichen thalli may assist the biocide absorption or, oppositely, that it may favour a quicker washing off and reduce absorption (Nugari and Salvadori, 2003; Pinna, 2017). Nevertheless, to the best of our knowledge, this issue has not yet approached experimentally. Similarly, it was hypothesized that the post-hydration may accelerate the action of the biocide (Tretiach *et al*., 2007). However, the practice of washing the treated surfaces some hours after biocide application to limit potential interferences with the stone substrate (Nugari and Salvadori, 2003) was never evaluated in terms of treatment effectiveness.

In this work, we aimed to verify the primary hypothesis that (a) biocide application tools, (b) pre-treatment hydration step, and (c) post-treatment washing may, either singularly or in combination, affect the effectiveness against lichens of biocides having different active principles and dilution solvents. In particular, the effectiveness of different biocide treatments against a foliose lichen, performed both in an archaeological site and in laboratory conditions, was tested in terms of chlorophyll *a* fluorescence of the thalli with respect to a vitality threshold (F_V/F_M = 0.15; Favero-Longo *et al*., 2017). We also verified the additional hypothesis that (d) stone substrate may act as reservoir for later biocide release under repeated cycles of wetting and drying.

5.2. Materials and Methods

5.2.1. Study site and lichen species

Biocide applications were performed, *in situ*, on the walls of the Amphitheatre of the Roman Archaeological site of Luni [Luni, La Spezia, Italy: UTM ED50, N 4879338, E 581882; 3 m a.s.l.]. Sandstone blocks of the Macigno Formation from Lunigiana were the main rock substrate. The Macigno Formation consists of fine to coarse sandstones with a variable degree of sorting that are mainly composed of quartz, feldspar and lithic grains (Franzini *et al*., 2007). Ripple cross-lamination locally occurs in fine grained samples. Carbonate cement is scarce and some clay may be present among grains.

Treatments were performed on the foliose lichen *Xanthoparmelia tinctina* (Maheu & A. Gillet) Hale, a species common from the submediterranean to the montane belt of Italy on siliceous rock surfaces, including the stone cultural heritage (Nimis *et al*., 1992). A total of 96 thalli were selected and treated *in situ* in April 2018 and May 2019. Lichen identification was performed in the field and checked in the laboratory following Giordani *et al*. (2002).

5.2.2. Biocide application in situ

Benzalkonium chloride (BAC) as 3% water solution of Preventol RI80 (alkyl dimethyl benzyl ammonium chloride, approx 80%, and isopropyl alcohol, 2%, in water; Lanxess, K¨oln, Germany), and N-octyl-isothiazolinone and 3-iodo-2-propynyl-N-butylcarbamate (OIT-IPBC) as 3% solution of BiotinR (OIT, 3-5%, and IPBC, 10-25%, in diethylene glycol butyl ether; CTS, Altavilla Vicentina, Italy) in white spirit (Kelix, Thormax

Italia, Roma) were selected as biocides. They were applied either (i) using a paint-brush or (ii) with a cellulose poultice (Arbocel BC 1000, JR Pharma, Rosenberg, Germany), (i') after having moistened the thalli with sprayed water or (ii') avoiding this pre-hydration step. Per each surface unit of thallus, brush applications required approx. 0.3 mL cm^{-2} of diluted biocides; the applied poultice layer, approx. 1 cm thick, contained approx. 12 mL cm^{-3}. The cellulose poultice was covered with a cotton fabric for 4 h and later gently removed with a small spatula, thereafter (i'') washing the thalli or (ii'') avoiding this washing step. Thalli treated with water only in place of biocides were assayed as negative controls. Three thallus replicates per biocide per application method were examined [i.e. 3 replicates × (2 biocides + 1 control) × 2 application tools × 2 pre-treatment approaches × 2 post treatment approaches]. Treatments including the pre-hydration step were performed in April 2018, and the others in May 2019. Bottled water with low mineral content (Fonti di Vinadio, Vinadio, Italy) was used as control, and for the biocide dilution and the pre-hydration and washing steps. Daily meteorological data (air temperature, relative humidity, rainfall) for the week prior and after the biocide applications in April 2018 and May 2019 were obtained from the nearby monitoring station of Luni (ARPA Liguria, 2018-2019; Fig. S1).

5.2.3. Biocide application in laboratory conditions

The application of BAC with the cellulose poultice was also tested in laboratory conditions. Treatment was performed on 14 thalli of *Xanthoparmelia* collected from a natural outcrop at Borgata Croux [Saint Cristophe, Aosta, Italy: UTM ED50, N 5068915, E 370323] together with their silicate (gneiss) substrate, avoiding any damage to Luni heritage

surfaces. Biocide application was performed on thalli with and without pre-hydration step (moistening with sprayed water). Seven replicates were performed for each condition.

5.2.4. Lichen vitality measurements

Chlorophyll *a* fluorescence measurements (Chl*a*F) - recognized as a tool for checking the vitality of photosynthetic organisms (Tretiach *et al*., 2008) - were carried out on *X. tinctina in situ* one day before (T0) and one day after (T1) biocide treatments, using a Handy-PEA fluorimeter (Plant Efficiency Analyser, Hansatech instruments Ltd., Norfolk, England). Analyses were performed on dark-adapted thalli, covered overnight with a black cotton fabric, which were moistened by sprayed water just before the measurements, to avoid that the additional hydration may further affect the biocide action. Measurements on the thalli treated in the laboratory were carried out one day before the biocide application (T0), immediately after the removal of cellulose poultice (T4h) and one day after (T1). Analyses were performed following the protocol adopted *in situ*, with the exception that the moistening at T0 was avoided for thalli foreseen without the pre-hydration step, and that measurements at T1 were performed for all thalli both before and after their moistening.

Five measurements were taken on each thallus, positioning the sensor head at 90° over its surface, inducing Chl*a*F by a red light (peak at 650 nm), and recording the data after a saturating light pulse of 1s (Malaspina *et al*., 2014). Chl*a*F increases from F_0, when all the reaction centres of PSII are open, to F_M, when all the reaction centres of PSII are closed. The maximum quantum efficiency of PSII, that is F_V/F_M (where $F_V = F_M - F_0$), a temperature-independent parameter of Chl*a*F emission, and variations in F_0, related to chlorophyll contents of the light

harvesting complex (Baruffo and Tretiach, 2007), were used to check the vitality of the thalli, in agreement with previous researches on the effectiveness of biocidal treatments against lichens (e.g. Tretiach *et al*., 2012; Favero-Longo *et al*., 2017).

5.2.5. Biocide absorption by lichen thalli

At the end of the fluorescence measurements at T1, the *X. tinctina* thalli treated *in situ* with BAC without performing the pre-hydration step, and the overall set of thalli treated in the laboratory, were gently detached from the rock substrate with a scalpel and processed to analyse the absorbed BAC. In particular, they were carefully cleaned under a stereomicroscope and then left overnight in a climatic chamber at 16° C and 55% of relative humidity (residual water content <10%). Samples of 50 mg were homogenized with 1 mL of deionized water and centrifuged at 20,000 rfc for 10 min. The supernatant was filtered at 0.45 µm using a syringe filter and 30 µL of the solution were directly analyzed by HPLC (Water LC I Plus). BAC was separated using a Phenomenex C18 (250 × 4.6 mm, particle size 5 µm) using a mixture of acetonitrile-sodium acetate buffer (pH 5.0; 0.2 M) (70:30, v/v) as mobile phase with flow rate 1 mL/min (Rojsitthisak *et al*., 2005). Runs were monitored at 210 nm. Quantification was performed with a calibration curve (5-50 µg/mL) of BAC from Sigma-Aldrich (≥95.0%). The limit of quantification of the analysis was 0.04 µg mg^{-1}.

5.2.6. Adsorption and desorption of benzalkonium chloride by sandstone lithologies

The property of different sandstone lithologies to adsorb and desorb BAC upon its application on the rock surface and a subsequent washing with deionized water was assessed in the laboratory. In particular, four sandstone blocks of the Macigno Formation, similar to those used in the Amphiteatre of Luni, were collected on the banks of the Parmignola, a stream located at few hundreds of meters from the Roman site, and cross sectioned with a diamond saw (section thickness >5 cm). In the central parts of the cut surfaces, parcels (2 × 3 cm) were established and treated with 250 µL of 3% BAC (Sigma-Aldrich, St. Louis, MO, USA), applied with a Transferpipette 100-1000 µL (Brand, Wertheim, Germany). The parcels were let to dry overnight at room temperature. Thereafter, 250 µL of deionized water were applied on each parcel and (after 30 s) a double layer of absorbent paper (9 mg cm 2) was applied -to simulate the potential absorption of a lichen thallus- and let dry on the rock surface. The absorbent paper was then suspended in 2 mL of deionized water to extract BAC, which was quantified as described above. Blocks of other sandstone lithologies employed in the Italian stone cultural heritage were also cross-sectioned and similarly processed for comparison, including the Pietra Serena, widely used in Tuscany (Fratini *et al*., 2014), the Cortemilia sandstone, from Southern Piedmont (Gelati *et al*., 2010), and the sandstone of the Verrucano Lombardo Formation, well known for rock-art in the Valle Camonica (Brack *et al*., 2008). At least three parcels per treatment (BAC, water) were considered per each block. Moreover, the same process was repeated on glass slides, as negative control.

Thin cross sections prepared from the rock blocks were observed by plane polarized light microscopy to characterize their mineral

composition and texture. Scanning electron microscopy in back scattered electron mode (SEM-BSE), undertaken with a JEOL JSM IT300LV (High Vacuum - Low Vacuum 10/650 Pa - 0.3-30 kV) and coupled with image analysis by the software WinCAM (Regent's Instrument, Canada), was used to estimate total porosity (Favero-Longo *et al*., 2009).

5.2.7. Statistics

Generalized Linear Models (GLMs) were applied to describe the effects of the different devitalization protocols on photobiont vitality *in situ* at T1, with the applied products (BAC, OIT-IPBC, and water as control), the application tools (brush and cellulose poultice), the pre-hydration and washing steps being considered as independent predictors. In particular, a factorial ANOVA analysis was performed to detect significant differences in F_V/F_M and F_0 according to the different predictors (product, application tool, pre-hydration, washing). GLM analyses were carried out with SYSTAT 10.2 (Systat Software Inc., San Jose, CA).

For all the analyses *in situ* and in the laboratory, significant differences in F_V/F_M at T1 between the different study cases and, for each study case, with respect to a viability threshold (set at F_V/F_M = 0.15, see Favero-Longo *et al*., 2017, with refs. therein) were analyzed by means of ANOVA with post-hoc Tukey's and *t*-test, respectively, using SYSTAT 10.2 ($P < 0.05$ as significant). For each study case, significant differences of F_0 in the thalli treated with biocides with respect to the control ones were assessed at T1. Significant differences in the absorption of BAC by lichen thalli, and in the BAC desorption patterns by different sandstone lithologies, were also examined by means of ANOVA with Tukey's post-hoc test.

5.3. Results

5.3.1. Efficacy of devitalization treatments in situ

GLM analyses (Table 1) showed that all the considered factors (product, application tool, pre-hydration and washing) contribute to determine the efficacy of devitalization treatments, evaluated in terms of F_V/F_M and F_0 of the targeted *Xanthoparmelia* thalli.

F_V/F_M values of thalli treated with biocides, independently of the application tool and the hydration protocol, were significantly lower than controls (Fig. 1). However, only in some cases values decreased below

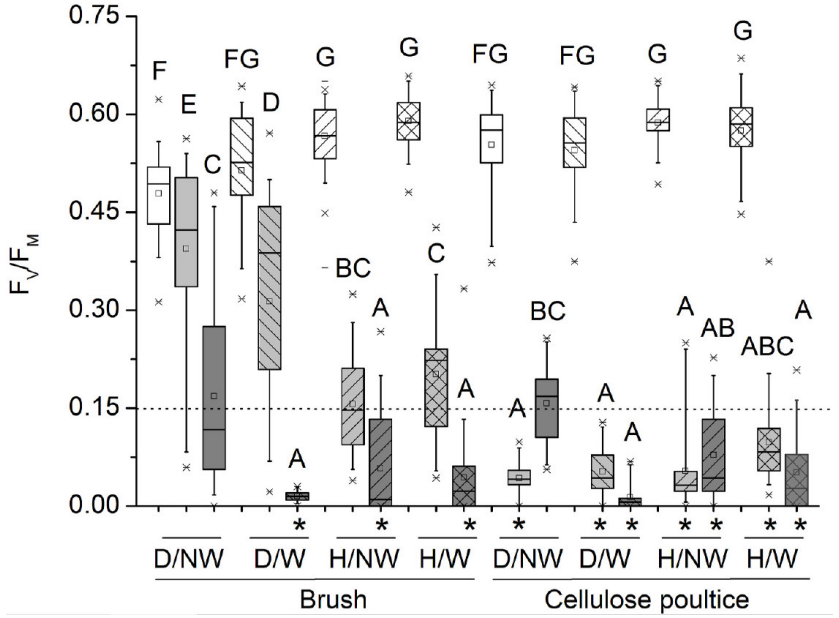

Fig. 1. Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of Xanthoparmelia tinctina *measured one day (T1) after the application, with brush (left box-plots) and cellulose poultice (right box-plots), of water (white box-plots; negative control), BAC (light grey) and OIT-IPBC (dark grey), coupled or not with pre-hydration (non pre-hydrated, D; pre-hydrated, H) and/or washing (non washed, NW; washed, W) of thalli. Box-plots which do not share at least one letter are statistically different (ANOVA, Tukey's test, $p < 0.05$). F_V/F_M values significantly lower than a viability threshold fixed at 0.15 (horizontal dotted line) are marked (*; ANOVA, t-test; $p < 0.05$).*

Table 1
Summary of the generalized linear model.

Parameter	Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
A) F_V/F_M	Product	32.029	2	16.015	196.125	**0.000**
	Appl. Tool	2.912	1	2.912	35.659	**0.000**
	Pre-Hydration	0.281	1	0.281	3.438	0.064
	Washing	1.659	1	1.659	20.317	**0.000**
	Error	55.852	684	0.082	-	-
B) F_0	Product	149018.955	2	74509.478	4.629	**0.010**
	Appl. Tool	248020.760	1	248020.760	15.410	**0.000**
	Pre-Hydration	99353.046	1	99353.046	6.173	**0.013**
	Washing	126628.182	1	126628.182	7.868	**0.005**
	Error	1.10090E+07	684	16095.073	-	-

the viability threshold (F_V/F_M = 0.15; Favero-Longo *et al*., 2017, with refs. therein). In particular, biocide application by brush was effective for OIT-IPBC, but only when coupled with thallus pre-hydration and/or post-treatment washing. Application with cellulose poultice was generally effective for BAC, while the effectiveness of OIT-IPBC was lower when thalli were not washed.

F_0 values (Figs. S2-S3) strongly decreased with respect to controls only for OIT-IPBC application on pre-hydrated thalli (mean ± SE: -62 ± 8%), in particular when thalli were not washed (–71 ± 9%). A relative increase of F_0 (144 ± 13%) followed all the applications of OIT-IPBC on non pre-hydrated thalli. BAC induced only slight decreases of F0 with respect to controls (–16 ± 3%).

5.3.2. Efficacy of devitalization treatments in the laboratory

In the laboratory, the application of BAC with cellulose poultice was effective against thalli moistened before

the treatment, while F_V/F_M of non pre-hydrated thalli did not significantly decrease beneath the vitality threshold of 0.15 (Fig. 2). In particular, fluorimetric measurements before the biocide application (T0) confirmed the well-known difference between the F_V/F_M of moistened thalli (ca. 0.7) and dry thalli (ca. 0.07). At the removal of the cellulose poultice (T4h), without any additional moistening, F_V/F_M of thalli treated in the wet state was significantly lower than the vitality threshold and with respect to thalli treated in the dry state. At T1, all thalli were dehydrated and F_V/F_M was significantly below 0.15, but after their moistening, those which had received the poultice application in the dry state recovered F_V/F_M values significantly higher than the threshold.

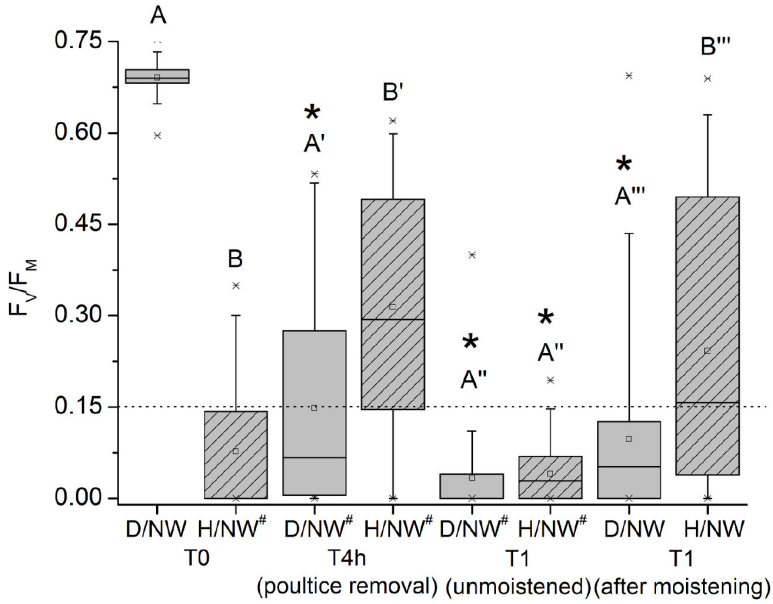

Fig. 2. Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of Xanthoparmelia tinctina *measured one day before the application of BAC with cellulose poultice (T0), immediately after the poultice removal (T4h) and one day after (T1), coupled or not with pre-hydration and/or post-treatment washing of thalli (codes as in Fig. 1). At each time point, box-plots related to thalli pre-hydrated (H) or not pre-hydrated (D) before the biocide application which do not share at least one letter are statistically different (ANOVA, t-test, $p < 0.05$). F_V/F_M values which are significantly lower than a viability threshold fixed at 0.15 (horizontal dotted line) are marked (*; ANOVA, t-test; $p < 0.05$). Thalli on which the fluorimetric measurements were performed avoiding the usual moistening step are indicated (#).*

5.3.3. Biocide content in lichen thalli

The absorption of BAC was detected and quantified in all lichen thalli treated *in situ* without performing pre-hydration, and in those treated in the laboratory, but concentrations strongly differed depending on the application method (Fig. 3). *In situ,* the thalli treated with cellulose poultice and not exposed to the final washing step displayed one order magnitude higher content of BAC (mean 1.4 µg mg^{-1}) with respect to those washed after the poultice removal and those treated with brush (0.1 µg mg^{-1}). In these latter, the BAC content was similarly low, irrespective whether the final washing was performed or not. In the laboratory, the content of BAC absorbed by thalli which were moistened before the application with cellulose poultice and not washed (mean 1.8 µg mg^{-1}) was similar to that detected *in situ* with the same application tool, but without pre-hydration. By contrast, the biocide content of thalli treated with cellulose poultice in the dehydrated state was significantly lower (0.2 µg mg^{-1}).

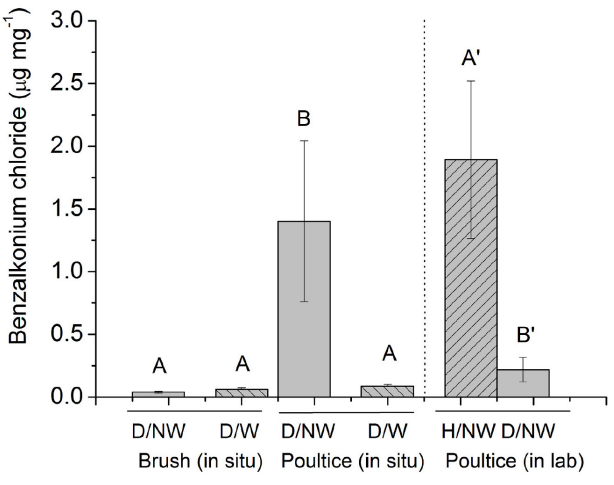

Fig. 3. BAC in thalli of Xanthoparmelia tinctina *after the application with brush and cellulose poultice* in situ *(four left columns) and with cellulose poultice in the laboratory (two right columns). Measures (mean ± SE) deal with non pre-hydrated thalli (D) and pre-hydrated thalli (H), which were washed (W) or not (NW) 4 h after the biocide application. Separately considering* in situ *and laboratory assays, bars which do not share letters are significantly different (ANOVA, Tukey's test, p < 0.05).*

5.3.4. Adsorption and desorption of benzalkonium chloride applied on sandstones

The amount of BAC desorbed from the rocks upon a re-wetting cycle, and thus absorbable by the absorbent paper used as to simulate the lichen thallus, was extremely low (always <0.5%; Fig. 4). The sandstone of the Verrucano Lombardo Formation showed a significantly higher desorption (0.34%), but remarkable differences were also detectable between the blocks of the Macigno sandstone, with values ranging from 0.15% (L2) to the detection limit (<0.03%; L1, L3, L4). The recovery of BAC from a glass slide (non-adsorbing substrate) was two order of magnitude higher, above 30%. On the basis of SEM-BSE observations (Fig. S4), the Verrucano Lombardo showed an intrinsic porosity remarkably lower than that of Macigno sandstone. Accordingly, BAC barely entered the rock volume and, upon the drying step, recrystallized directly on the surface, from which it was mobilized during the subsequent re-wetting. Oppositely, in the case of the other sandstones, the applied biocide clearly entered the rock volume. In the case of the Macigno sandstones, microscopic observations of petrographic thin cross sections showed that a clay fraction occurred in L1, L3 and L4, while it was absent in L2 (Fig. S5). Pietra Serena showed a fitted fabric due to pressure dissolution, with juxtaposed grains and absence of cement or matrix, while the Cortemilia sandstone showed traces of carbonate cement and a clay fraction. A fine-grained sericitic matrix possibly characterized the block of Verrucano Lombardo.

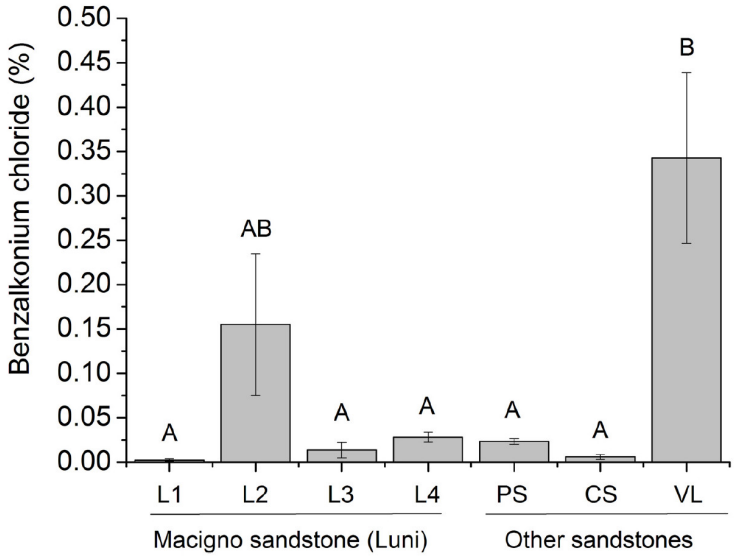

Fig. 4. BAC absorbed by absorbent paper -used to simulate a lichen thallus- after its desorption from the Macigno sandstone, used in the Amphitheatre of Luni, and from other sandstones for comparison (Pietra Serena, PS; sandstone of Cortemilia, CS; sandstone of the Verrucano Lombardo Formation, VL). Data are expressed as percentage of the amount of benzalkonium chloride (7.5 mg) initially applied on the examined parcels (mean ± SE). Bars which do not share letters are significantly different (ANOVA, Tukey's test, $p < 0.05$).

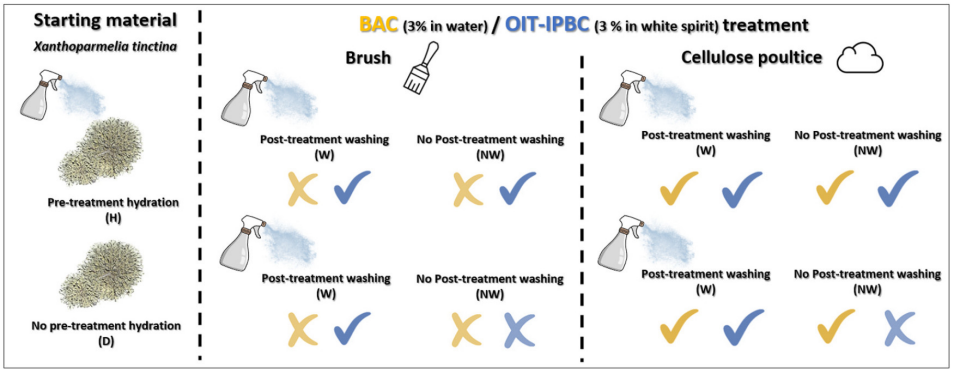

Fig. 5. Synoptic comparison of the influence of different application protocols on the efficacy of biocide treatments against the foliose lichen Xanthoparmelia tinctina *(F_V/F_M at T1 was, ✓, or was not, ×, significantly lower than the vitality threshold set at 0.15).*

5.4. Discussion

Our findings support the hypothesis that the protocol adopted to apply biocides significantly affects the devitalization of lichen thalli (Fig. 5). Besides confirming the importance of the application tool, with cellulose poultice being more effective than brush (Favero-Longo *et al*., 2017; Matteucci *et al*., 2019), this experimental work clarified the remarkable influence of the state of hydration of lichen thalli on their susceptibility to biocides. Hydration modulates the biocide absorption by thalli. Moreover, it controls their maintaining an active metabolism or entering a dormancy state, thus succumbing to or tolerating, respectively, the available toxic compounds. In relationship with the water or organic solvent preparation of the assayed biocides, we highlighted the biocide-specific advantage of pre-treatment hydration and/or post-treatment washing of thalli to improve the application protocol effectiveness. In particular, the poultice application was necessary to make effective against *X. tinctina* the assayed water-solution of benzalkonium chloride (BAC), independently of the pre- or post-treatment hydration of thalli. Differently, the washing of thalli after the biocide application was necessary to make effective the assayed organic-solvent solution of N-octyl-isothiazolinone and 3-iodo-2-propynyl-N-butylcarbamate (OIT-IPBC), either applied by brush or with cellulose poultice.

In this regard, until innovative strategies to control biodeteriogens will be routinely available, the conventional use of traditional biocides by restorers cannot overlook this necessity of adopting effective application protocols and hence limit the useless release of biocides in the environment. In addition, this work showed that the stone substrate, depending on the lithology, may variously absorb the applied biocide, potentially contributing as a reservoir for its long-term release at low

concentrations during successive hydration events.

5.4.1. Biocide efficacy and thallus hydration

Lichen tolerance of extreme stress conditions is well documented and has been related to their ability to cyclically enter and leave a dormancy state by thallus dehydration and rehydration, respectively (Beckett *et al*., 2008). Such adaptation is supported by enzymatic and non-ezymatic mechanisms to protect the integrity of cellular components and limit pro-oxidative processes (Kranner *et al*., 2008), an effective machinery to maintain proteostasis (Armaleo *et al*., 2019) and the interplay of the whole lichen microbiota (Cernava *et al*., 2019). A notable example is the tolerance to high temperatures, which for dry thalli ranges from 70°C to more than 100°C depending on species (Lange, 1953), while it is generally lower than 45-50°C when thalli are forcedly maintained in the hydrated state (McFerlane and Kershaw, 1978; Tretiach *et al*., 2012). In agreement, lichen resistance to gaseous pollutants, as SO_2 and O_3, is higher during the dry state; by contrast, the pollutants can dissolve in the hydrated thallus, in which the symbionts are metabolically active and sensitive to their toxic effects (Vannini *et al*., 2020).

A similar pattern is here confirmed for the foliose lichen *X. tinctina* treated with the water soluble BAC and OIT-IPCB prepared in white spirit. The quaternary ammonium salt BAC perturbs the phospholipid bilayer of the biological membranes, causing their damage and the cell lysis (Wessels and Ingmer, 2013). OIT oxidizes thiol-containing cytoplasmic and membrane-bound compounds, yielding metabolic inhibition (Denyer and Stewart, 1998), and IPBC disrupts the formation of fungal cell walls by interfering with synthesis of phospholipids and fatty acids (Biehl, 2019). Despite their different active principles, target molecules and

solubility, thallus hydration influences the effectiveness of both products.

The poultice application of BAC *in situ*, which always decreased F_V/F_M below the viability threshold set at 0.15, carried the water-dissolved biocide as well as contributed to maintain wet the pre-hydrated thalli and to hydrate the thalli in the dry state (Favero-Longo *et al*., 2017). Such latter effect was clearly recognizable in the laboratory assays, in which the very low F_V/F_M of the initially dry thalli remarkably increased at the time of the poultice removal (T4h), indicating its metabolic activation by water rather than its devitalization by the biocide. The dry state at the time of the application also implied in the laboratory a significantly lower BAC absorption with respect to that detected in the pre-hydrated thalli. Such findings agree with the linear positive correlation between the hydration of thalli and their efficiency to accumulate persistent organic pollutants (Kylin and Bouwman, 2012; Augusto *et al*., 2012) and reject the hypothesis that pre-hydration may reduce the absorption of biocides (Tretiach *et al*., 2007).

In situ, BAC absorption in non pre-hydrated thalli was instead more similar to that of the pre-hydrated thalli in the laboratory. With this regard, it is worth noting that F_V/F_M of lichen thalli, and thus their metabolic activity, is also highly related to weather conditions during the two days prior to the measurements (Vivas *et al*., 2017). Differing from the thalli kept in the laboratory in the dry state, those in the study site were regularly exposed to high humidity levels during the night (RH above 80-90% in the days before the treatment; Fig. S1). Although they were dried at the time of biocide application (around noon), they likely had a higher attitude, in terms of physiological state, to recover their metabolic activity by effectively absorbing the water solution of BAC, even without the pre-treatment hydration step.

The application of BAC by brush contributed a lower quantity of biocide and did not maintain the hydration of the thalli, justifying a lower

absorption and the poor devitalization effectiveness. The fact that post-treatment washing did not lead to a further lowering of F_V/F_M suggests that the effect was likely more limited by the biocide quantity than by a scarce metabolic activation. This also agrees with the fact that the absorbed BAC did not decrease with the post-washing step, suggesting that the available biocide had been effectively absorbed and retained by the cell structures. In this sense, neither brush nor poultice application determined at T1 a remarkable decrease of F_0, detectable upon the loss of chlorophyll following membrane integrity impairment (Vannini *et al*., 2018), suggesting that BAC-driven cell lysis had still not deeply proceeded and that some absorbed BAC could not be washed away.

In the case of OIT-IPBC, no difference was detected in the effectiveness of the assayed application tools, indicating that the lower quantity of active principles carried by the brush was sufficient to kill the lichens. However, for this biocide prepared in white spirit, the wetting of thalli by the pre-treatment hydration and/or the post-treatment washing was a necessary requirement to make the treatment effective. Accordingly, the removal of crustose and foliose lichens following the application of BiotinR with a protocol which does not mention hydration steps determined the persistance of thallus remains with some (few) viable photobiont cells (de los Ríos *et al*., 2012). As hypothesized for other biocides, but not experimentally verified (Tretiach *et al*., 2007), the post-treatment washing of thalli treated when dry showed the highest effectiveness in the F_V/F_M decreasing, suggesting that the lichen recovery of the metabolic activity in the presence of the toxic molecules was the most suitable method to favour its susceptibility and face its defence strategies. This agrees with the report that IPBC is highly soluble in organic solvents and poorly soluble in water (156 mg L^{-1} at 20°C; Juergensen *et al*., 2000), but its efficacy depends on the water dissolved

fraction and its general wide use is related to strategies to allow its dissolution, including the predissolution in organic solvents (Steinberg, 2002). However, the highest decrease of F_0 (>60%) was observed in thalli pre-hydrated, either washed or unwashed, suggesting that they mostly faced a strong damage of cell structures and the damage and loss of chlorophyll. In thalli treated when dry, instead, F_0 showed a relative increase, which may reflect the initial presence of some free chlorophyll due to membrane damage (Strasser, 1997), or a resistance attempt towards a treatment with incomplete killing efficacy (Favero-Longo *et al*., 2017). Further investigations will be necessary to clarify such response patterns of thalli as well as to unveil if and how different application protocols may also variously impact the hyphal penetration component of lichens (*sensu* Favero-Longo *et al*., 2005) and the associated microbial communities, which already revealed different sensitivity to different biocidal products (de los Ríos *et al*., 2012) and play a crucial role in biodeterioration processes (Speranza *et al*., 2012).

5.4.2. Does substrate porosity influence the biocide efficacy?

The different effectiveness of biocides against the growth of algae inoculated on sandstone lithologies was related to their different porosity and clay contents (Young *et al*., 1995). Biocides can penetrate below the surface and either be bio-available while bound to the minerals or be slowly desorbed and become available to (micro-)organism absorption under repeated cycles of wetting and drying (Cameron *et al*., 1997). For some biocides, the adsorption to clay minerals may determine their inactivation, but quaternary ammonium salts should maintain their biocidal activity when bound (Walters *et al*., 1973; Cameron *et al*., 1997). These processes, however, have been

infrequently investigated (Koestler and Salvadori, 1996) and their consequences for practical issues of restoration protocols -such as their effect on recolonization dynamics- are scarcely taken into account. In particular, they should be carefully considered with respect to the widespread application of biocides as a preventive tool to protect heritage surfaces from recolonization process, which is to maintain rock cleaning after the removal of lichens and biofilms (Pinna, 2017). In agreement with previous works (Young *et al*., 1995; Cameron *et al*., 1997), our laboratory experiment showed that the amount of BAC desorbed by a wetting event, following the biocide application and consequent rock adsorption, depends on physical and mineralogical properties of the different sandstone lithologies. In particular, the higher the rock porosity and the presence of clay minerals, the lower the biocide desorption at new rain events or watering. In the case of lithologies with very low porosity, such as Verrucano Lombardo, the biocide visibly crystallized at the rock surface and could likely be washed off by flowing water rather than persist as long-term protection (Cameron *et al*., 1997). Even within the same lithology, clay contents can vary from a block to another, as in the case of the Macigno sandstone in Luni, and thus differently affect biocide adsorption and desorption. For all the examined lithologies, the amount of desorbed biocide potentially available to microbial absorption is 2-3 orders of magnitude lower than that provided during the application, which turned effective only in the case of the copious poultice treatment. Accordingly, the biocides applied after the cleaning interventions may possibly exert their preventive protection insofar they remain abundantly bound within the rock porosity (Cameron *et al*., 1997), although the bio-activity should be demonstrated for each considered quaternary ammonium compound. On the other hand, such application strategy produces a reservoir for their gradual release at low and likely ineffective concentrations. With

this regard, the phenomenon may be likely related to the reported cases of surface eutrophication following the application of quaternary ammonium salts, their degradation and consequent nitrogen supply, favouring recolonization processes by nitrophilous, fast growing species (Scheerer *et al*., 2009). Moreover, the release of low and ineffective concentrations of BAC can promote bacterial adaptation and antibiotic resistance (Kampf, 2018; Kim *et al*., 2018; Poursat *et al*., 2019).

5.5. Conclusions

This work confirmed the hypothesis of a biocide-specific importance of the application tools, the pre-treatment hydration and/or the post-treatment washing to make the devitalization treatments effective against lichens (F_V/F_M of lichen thalli after the treatment <0.15).

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(following the format style of International Biodeterioration and Biodegradation)

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5.6. Supplementary materials

Fig. S1. Meteorological parameters during the week preceding the biocide applications (D-7/D-1), during the days of the biocide application (DT), and the days after (D+1), when fluorescence measurements at T1 were performed. Biocide applications (DT): 19 April 2018, 12 pm (black line); 21 May 2019, 12 pm (red line).

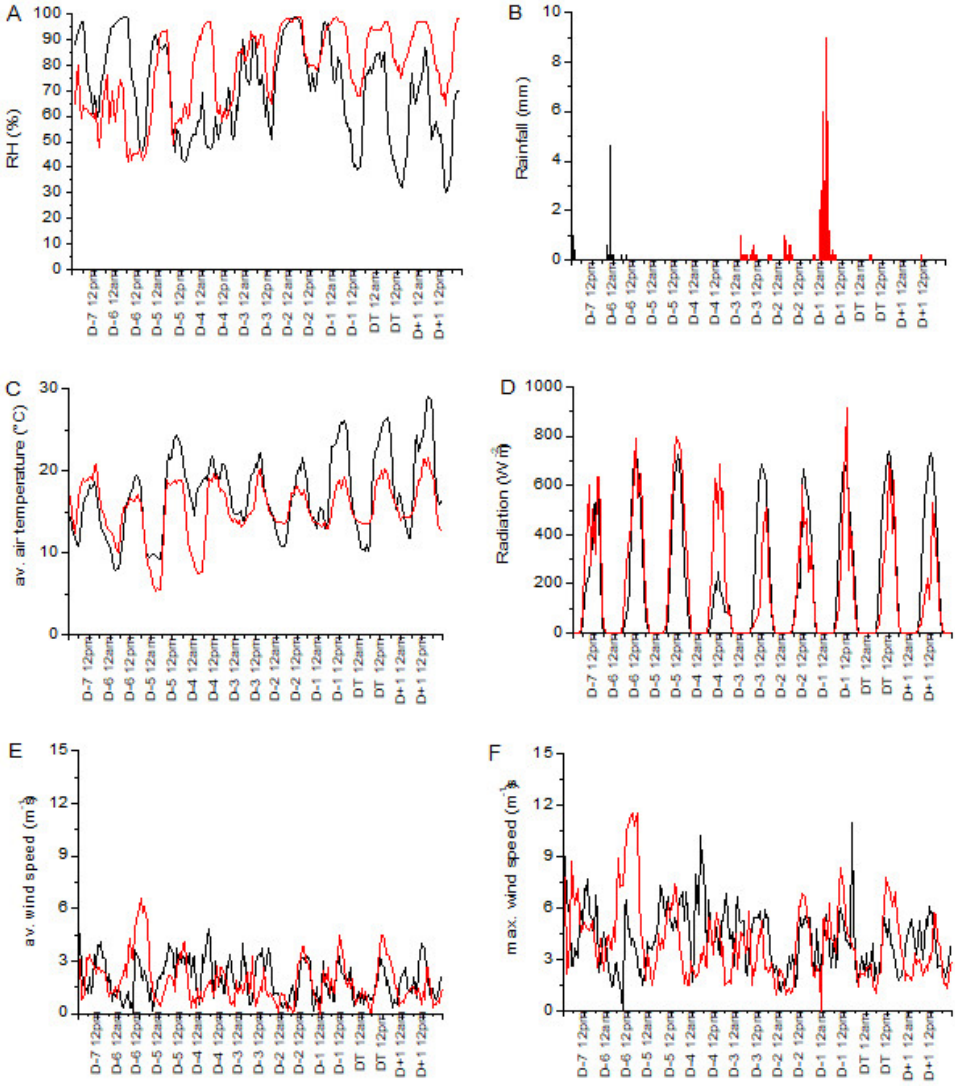


Fig. S2. Difference of F_0 values (Δ%) in thalli of Xanthoparmelia tinctina *measured one day (T1) after the application with brush (left box-plots) and cellulose poultice (right box-plots) of BAC (light grey box-plots) and OIT-IPBC (dark grey), coupled or not with pre-hydration and/or washing (codes as in Fig. 1), with respect to related control thalli treated with water only. Absolute values of F_0 are shown in Fig. S3.*

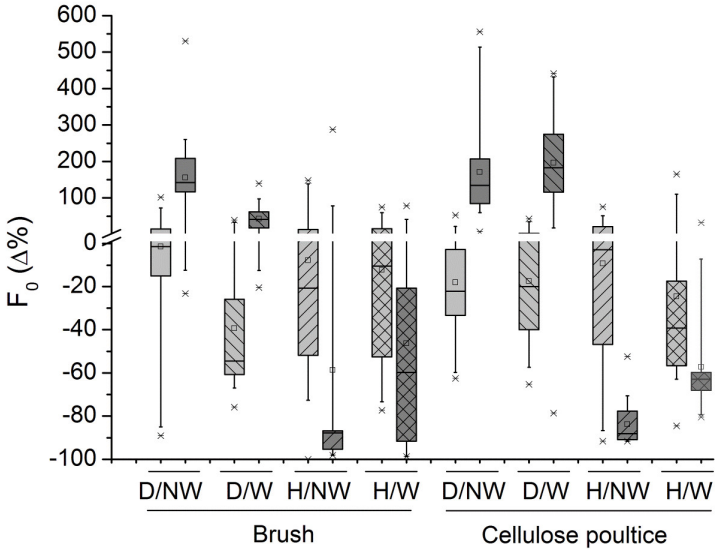


Fig. S3. F_0 values in thalli of Xanthoparmelia tinctina *measured one day (T1) after the application with brush (left box-plots) and cellulose poultice (right box-plots) of water (white box-plots; negative control), BAC (light grey) and OIT-IPBC (dark grey), coupled or not with pre-hydration (non pre-hydrated, D; pre-hydrated, H) and/or washing (non washed, NW; washed, W) of thalli. Box-plots which do not share at least one letter are statistically different (ANOVA, Tukey's test, $p<0.05$).*

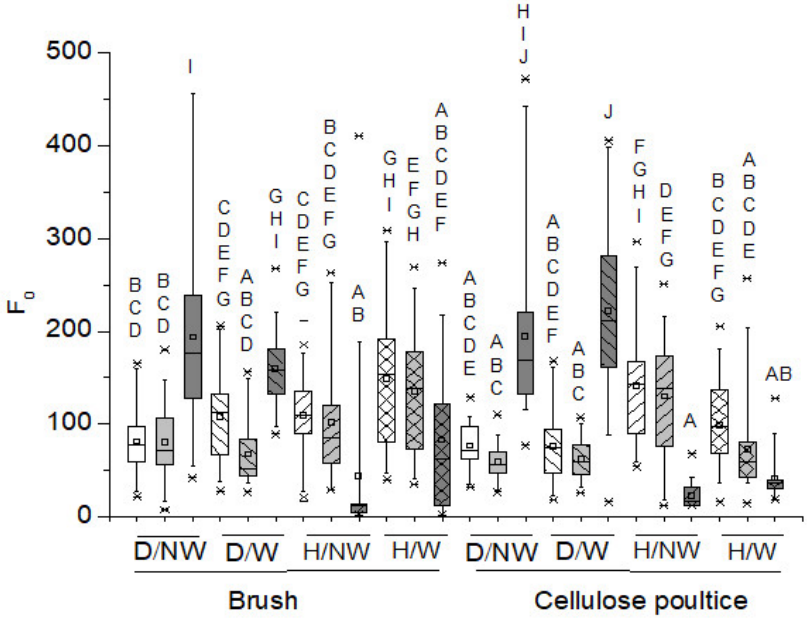


Fig. S4. Porosity of sandstones as seen in thin cross-sections observed under scanning electron microscopy in back-scattered mode (SEM-BSE). Black areas correspond to intrinsic porosity between the rock-forming minerals (grey and white coloured areas) of the Macigno sandstone (L1-L4; A-D), Pietra Serena (PS; E) and the sandstone of Verrucano Lombardo Formation (VL; F). Scale bars: 1 mm. These representative images were analyzed using the software WinCAM Pro 2007d (Regent's Instruments) to estimate porosity, as follows: L1 9.1%, L2 4.9%, L3 1.93, L4 5.7%, PS 0.7%, VL 0.6% (SEM images and porosity data are not available on the sandstone of Cortemilia).

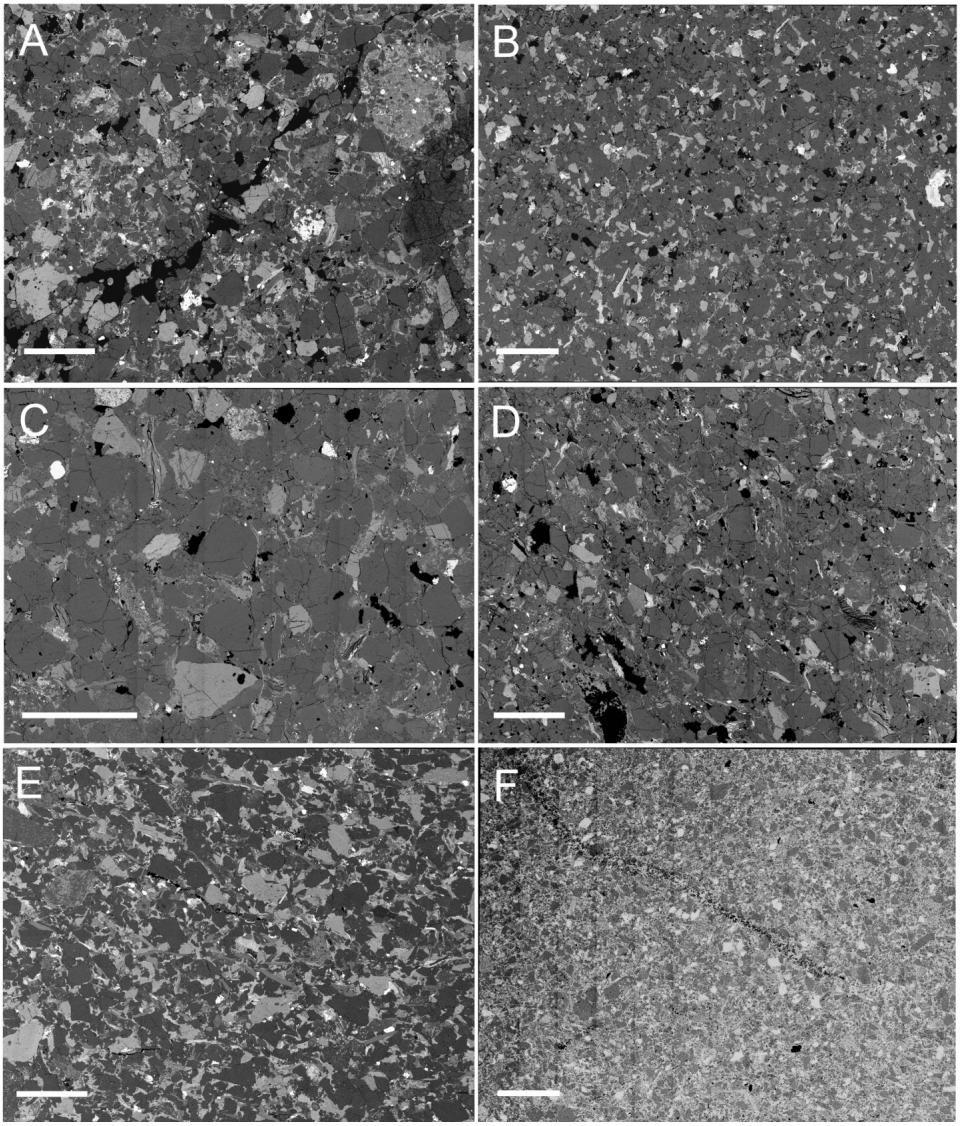
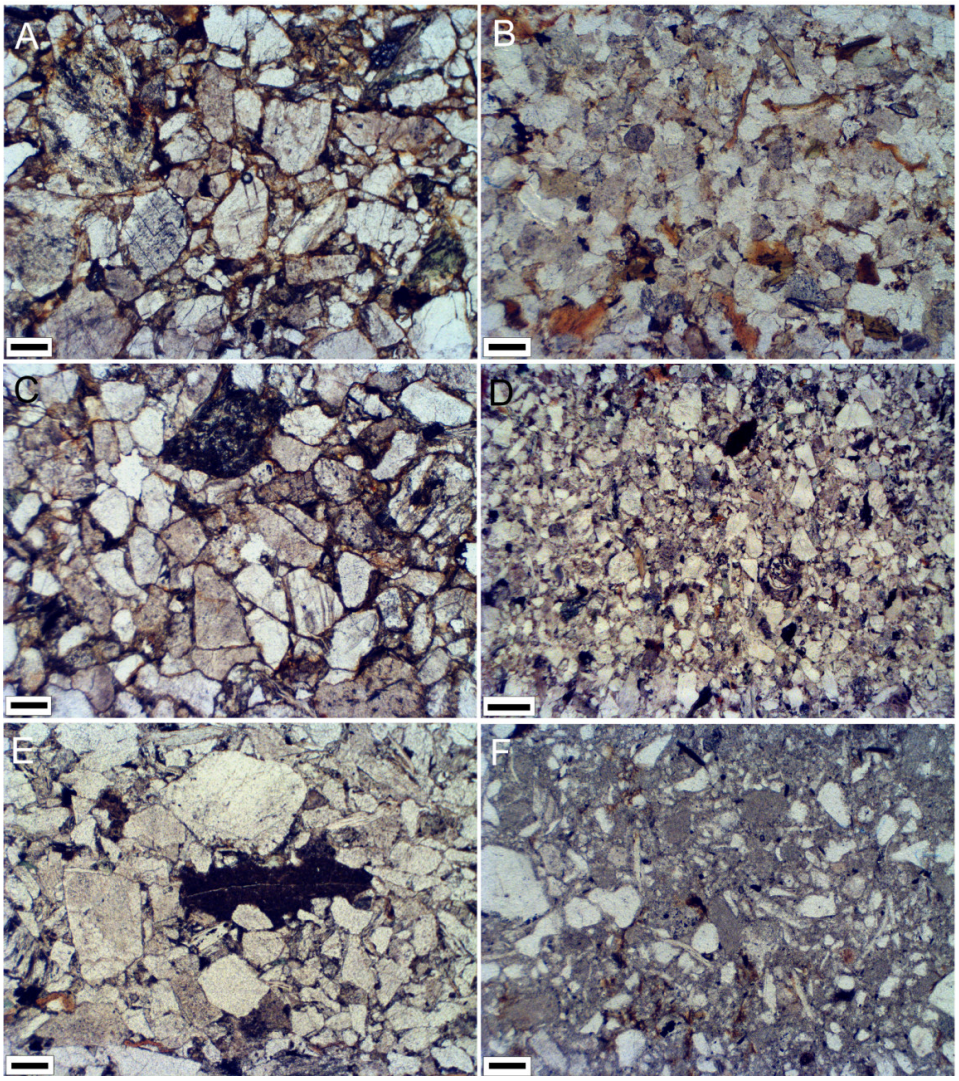


Fig. S5. Macigno Formation (L1-L3; A-C): medium to fine grained, quartz and feldspar-rich sandstones; minor mica flakes are locally recognizable. Grains are very tightly packed with long and also concavo-convex boundaries which document an intense mechanical and chemical compaction. Cortemilia Formation (CS; D): poorly sorted sandstone with very fine to medium sized grains composed of quartz, feldspar, mica flakes, lithic grains of metamorphic rocks, and carbonate grains locally consisting of bioclasts. Long and some concavo-convex boundaries among grains show mechanical and chemical compaction. Some minor portions of carbonate cement are recognizable. Pietra Serena (PS; E): poorly sorted sandstone with quartz, feldspar and mica grains. A dark mud clast is recognizable in the centre. The penetration of the surrounding grains within the clast shows that it was in a plastic state at the time of deposition of the sand. Concavo-convex grain boundaries, documenting pressure dissolution, are particularly common. Verrucano Lombardo Formation (VL; F): fine grained sandstone with quartz grains, mica flakes and abundant grey lithic fragments of the groundmass of volcanic rocks. Thin section, plane polarized light. Scale bar: 200 µm (A-C, E, F), 500 µm (D).



Chapter 6

Conclusive remarks and future perspectives

In this PhD thesis, the value and relevance of biological analyses for biodeterioration studies of outdoor stone surfaces, and related topics, have been confirmed: scientific analyses on the pattern and mechanisms of lithobiontic colonization contribute to address conservation management, while a better knowledge on the efficacy of biocidal treatments may drive the choice of the best growth control strategies.

The influence of **micro-environmental conditions on biodiversity (A)** was detailed in the study of lichen colonization on the vertical walls in the archaeological site of Pompeii (Chapter 2). Parameters related to the material and the architectural geometry were established as proxies of different micro-environmental conditions, and some of them were found more relevant in driving the composition of the lithobiontic communities. The results of this study could easily be applied in the whole archaeological area and other stone monuments characterized by analogous regional climate. Moreover, the method of investigation can be replicated in different climates, on different materials, situations, on various types of architectural geometries and on different (micro-)organisms. The consequences of this type of study fall back in the assessment of the best preventive growth control strategies, aimed to avoid or limit stone colonization by the more deteriorating communities (Caneva *et al*., 2016; Traversetti *et al*., 2018). For example, in the study in the site of Pompeii, the distance from the ground, related to a lower water availability and greater exposition to sunlight and to

propagule deposition, was one of the most important parameters driving the colonization of the biodeteriogenic lichens *Verrucaria nigrescens* and *Dirina massiliensis*. This information indicates a not obvious influence of water availability on the biodeterioration risk, while sun exposition might be more relevant. As a consequence, in a similar case, the biology expert could suggest a realization of local interventions to protect specifically the higher, more exposed portions of the historical walls to focusly prevent the colonization by lichen communities, such as preventive treatments with biocides/allelopathic substances, or periodic wiping to limit propagule establishment, or even -if compatible with respect to other issues- structures shielding from sunlight.

In the study reported in Chapter 5, the influence of biocide treatments on lichens is evaluated in relationship with different parameters related to the material, the method of biocide application and the thallus hydration. This last parameter is again related to (micro-)environmental conditions: for example, in an archaeological site, a shaded wall could be a source of water availability, while another one exposed to sun could be more dry, suggesting different levels of criticality in the two cases (Nimis *et al*., 1998).

Moreover, in the study on the biocidal effect, stone properties were related to a different absorption of biocide, potentially acting as a reservoir of biocidal substance and thus further delaying the re-colonization. However, biocidal substances are soluted in different solvents which can be differentially absorbed in stone porosity. For these reasons, the assessment *in situ* of the best biocidal treatment should keep into consideration the efficacy on species, the effect of biocide on stone and *viceversa*, the best hydration condition based on the solvent of the biocide and the environmental conditions which interact with the

process.

The importance of the knowledge of the **physical and chemical space of interaction of lithobionts with their substrate** has been explored in a large number of studies (e.g. Crispim and Gaylarde, 2005; Favero-Longo *et al*., 2011; Marvasi *et al*., 2012; Salvadori and Casanova-Municchia, 2016), but information on the relationship of microorganisms with stone is still incomplete. Microbial interaction with stones depends on the species, the material and the external conditions (Pinna, 2017), which draw the attention towards the high variability of situations and, consequently, the different effects in terms of biodeterioration. In the study on the involvement of melanin in the active penetration of the microcolonial fungus *Knufia petricola* within carbonate pellets (Chapter 3), the thin, filamentous growth form, poorly studied for lithobiontic fungi, was identified, and its penetration pattern was investigated and compared to that of the more studied torulose hyphae. The hyphal staining highlighted the presence of filamentous hyphae deep in the pellets, exploiting the porosities of the material with more efficiency than torulose hyphae, due to the smaller hyphal diameter. This deep endolithic colonization could be more resistant to biocidal and removal treatment, potentially decreasing their efficacy (de los Ríos *et al*., 2012). Moreover, fungal growth was often observed as a (re-)colonization phenomenon after biocidal or consolidation treatments (Bastian *et al*., 2010). In particular, the consolidants can be used to decrease stone bioreceptivity by sealing the porosities and thus lowering water availability (Pinna, 2017), but, if microcolonial fungi can use consolidants as a nutritional source and, with filamentous hyphae, exploit the pre-existing porosities, the consolidation of surface would be detrimental (Favero-Longo *et al*., 2018). However, the presence, pattern and

penetration of filamentous hyphae has not been studied in the field, and not even on natural stone coupons. A next step of this study has already been launched, aimed to analyse the presence of filamentous hyphae and melanized torulose hyphae in natural marble samples and on other silicatic lithologies. A subsequent step would be the study in samples collected in natural environment of microcolonial fungi penetration with particular attention to the filamentous hyphae, to assess if its presence can only be massively detected in *in vitro* oligotrophic, but UV-protected, conditions, or if this growth form can also be found in nature. This would also improve the knowledge on lithobiontic microcolonial fungi ecology, which is not completely clarified yet (Coleine *et al*., 2021). Moreover, the biodeterioration potential of filamentous hyphae has not been fully assessed. Further research should be aimed to determine if this thin growth form just exploits the porosity, causing no damage, or if it could cause chemical biodeterioration and/or lead to the formation of melanized torulose hyphae, more invasive both aesthetically and structurally.

The study of lichen influence on stone hardness (Chapter 4) also explored the lithobiont-substrate interaction. Many studies have already observed the extensive ability of lichen hyphae to penetrate within stone surfaces (e.g. Favero-Longo *et al*., 2011; Guglielmin *et al*., 2011, McIlroy de la Rosa *et al*., 2013), particularly evident in porous materials such as sandstones (Bartoli *et al.*, 2014), which was confirmed in this study. However, the data on stone hardness variation, which differed particularly between *Verrucaria muralis* with respect to the other two species considered, was unexpectedly non-related to hyphal penetration depth, which led to a deeper research in the other possible interaction mechanisms between lichen species and sandstone. This analysis, based

on a XRPD approach, produced preliminary data on the species-specific biomineralization patterns, which should be further explored to determine the role of lichens in calcite re-precipitation, the possible involvement for stone conservation and their implication in geomorphological processes.

The **overall effect of lithobiontic colonization on stone durability, meant as a balance between biodeterioration and bioprotection impacts**, depends on a variety of parameters all intertwined together (e.g. McIlroy de la Rosa *et al*., 2013; Bartoli *et al*., 2014). Maybe due to the complexity of the situation, researchers have scarcely confronted this issue, even though this equilibrium should drive the considerations regarding conservation strategies (Favero-Longo and Viles, 2020). In the study in Chapter 4, the effect of lichen colonization on sandstone hardness has been measured and related to some lichen-substrate interaction mechanisms, to determine if the effect of hardness could be directly correlated to one of these patterns. Interestingly, biochemical interaction of calcite bio-precipitation seemed to be more involved than physical one. This result prompts interesting lines of research which could deepen the mechanisms of lichen biomineralization, scarcely studied until now. A biomineralized surface presents fine, micritic calcite which deposits in stone porosity (Garvie *et al*., 2008) and could therefore be involved in bioprotective mechanisms (Bungartz *et al*., 2004). Moreover, the area of influence of lichen colonization into the stone could be wider and deeper than hyphal penetration, and could include the effects of lichen-derived molecules, as proteins and lipids, involved in bioprecipitation and, possibly, hardening processes (see Tonon *et al*., 2021 in Appendix 3).

All of the different topics faced in this PhD thesis, and resumed up to this point, propose, as a final outcome or as a consequential perspective, hints and suggestions on the growth control strategies for stone surface conservation. Additionally, in chapter 5, the focus on growth control is more straight, delving into the **efficacy of biocidal treatments depending on selected substances, method of application and environmental conditions**. The influence of hydration, related to (micro-)environmental conditions, the biocides and their solvents, and the material and the species involved were all determined as relevant factors in the assessment of the most efficient biocidal treatment on outdoor stone surfaces. Practical information about these topics can be sometimes found in literature, but rarely organized and uniform and, maybe for this reason, are often neglected when conservators plan a removal of biological patinas (Favero-Longo *et al*., 2017). Nevertheless, being the chemical biocidal treatments the most diffused, the importance of a accurate knowledge on the correct quantity and method of treatment is evident, to avoid the use of overabundant quantities of biocides, which could be washed-away, endangering the environment and ultimately being ineffective at devitalizing lithobionts (Pinna, 2017).

In the same way, it is important to avoid the application of sub-inhibitory doses of biocidal compounds, since this could cause effects of resistance to biocides (Berman and Krysan, 2020). For example, the use of sub-inhibitory concentration of benzalkonium chloride has been proved to induce a reduction in sensitivity, which can lead to a rapid emergence of extremo-tolerant microbial communities (Mc Cay *et al*., 2010; Kim *et al*., 2018). This is a known phenomenon in the healthcare and food industry fields (Tezel and Pavlostathis, 2015), but few studies in the stone conservation field dealt with this issue, and more analyses should aim to contribute to the definition of precise protocols to follow

when removing lithobiontic colonization. The effectiveness of the treatment needs to be first assessed in standardised conditions in laboratory on a single organism and then on controlled association of a few microbial species (Vannini *et al*., 2018), and subsequently experimented in the field, to confront with the high number of variables participating in real cases (Gorbushina *et al*., 2003; Pinna, 2017).

The assessment of correct biocidal treatments would be useful also on microorganisms with interaction patterns with the stone not clarified yet, such as the filamentous hyphae of *Knufia petricola*. This growth form, whose development was related to a situation of scarcity of nutritional substances, penetrated the substrate more deeply than the torulose, melanised hyphae, and it is possible that a biocide would fail to reach and devitalise the more deep structures. In this case, the filamentous hyphae could represent a mechanism of resistance in the field, if the biocidal treatment is not carefully calibrated.

Some considerations on natural biocides are reported in a side-work (Appendix 1), giving a glimpse on the rising relevance that the research of alternative growth control strategies is acquiring. In particular, the persistence of lichen secondary metabolites was hypothesized on the marble of the *Caestia* Pyramid in Rome, matching with the visual observation of round thallus-like tracks where the growth of black patina seemed to be inhibited. A permanence of lichen secondary metabolites may be indeed related to an allelopathic effect towards the microorganisms of the black patina, as previously verified in laboratory assays (Gazzano *et al*., 2013). Further analyses on this and alternative hypotheses to explain localized inhibition of black biofilms on the stone cultural heritage are now in progress (Favero-Longo *et al*., 2021 in Appendix 4), also aiming to evaluate their actual potential as alternative

biocidal substances, as recently proposed for many plant-derived compounds, as essential oils (Fidanza and Caneva, 2019). However, even if all these substances have a natural origin and a proven toxicity on microorganisms, they could also represent a threat for the environment, leading to a necessity of studies considering the interaction among new, natural substances and the environment (Cappitelli and Villa, 2021).

An additional promising alternative may be the treatment of surfaces to prevent microbial colonization by making the material less bioreceptive. Polymeric consolidants, antifouling nano composites, titanium oxide -among other products- were studied for application for stone conservation (Lo Schiavo *et al*., 2020). In Chapter 4, lichen biomineralization processes were analysed and preliminarily related to possible bioprotection effects. If this mechanism will be confirmed and characterized with a joint mineralogical and biomolecular approach, already in progress, a chance for an upgraded use of bioprecipitation of calcite in the field of conservation. In particular, such improved knowledge would prompt the application of molecules and biogeochemical processes involved in bioprotection, but avoiding the negative effects related to the lichen and microbial growth and their physical impacts.

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Appendices

sustainability

MDPI

Article

Biodeterioration Patterns and Their Interpretation for Potential Applications to Stone Conservation: A Hypothesis from Allelopathic Inhibitory Effects of Lichens on the *Caestia* Pyramid (Rome)

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Abstract: The colonisation of stone by different organisms often leaves biodeterioration patterns (BPs) on the surfaces even if their presence is no longer detectable. Peculiar weathering patterns on monuments and rocks, such as pitting phenomena, were recognised as a source of information on past colonisers and environmental conditions. The evident inhibition areas for new bio-patinas observed on the marble blocks of the *Caestia* Pyramid in Rome, recognisable as tracks of previous colonisations, seem a source for developing new natural products suitable for restoration activities. To hypothesise past occurring communities and species, which gave rise to such BPs, we carried out both in situ observations and analyses of the rich historical available iconography (mainly photographs). Moreover, we analysed literature on the lichen species colonising carbonate stones used in Roman sites. Considering morphology, biochemical properties and historical data on 90 lichen species already reported in Latium archaeological sites, we suppose lichen species belonging to the genus *Circinaria* (*Aspicilia* s.l.) to be the main aetiological agent of such peculiar BPs. These results seem relevant to highlight the long-lasting allelopathic properties of some lichen substances potentially applicable as a natural product to control colonisation, improving the environmental and economical sustainability of stone restoration.

Keywords: lichen allelopathic substances; natural biocides; stone biodeterioration; stone conservation; historical photographs

1. Introduction

Stone monuments are affected in different ways by organisms and microorganisms, whose metabolic activity and growth can impact them aesthetically, chemically and/or physically, determining biodeterioration phenomena [1–6]. Sometimes their colonisation can also exert a positive bioprotective action, as shown for some monuments all over the world [7–9]. In particular, despite the deteriogenic interactions, their covering can lower the weathering by external environmental factors (e.g., winds, low temperatures, rainfalls) when these are very aggressive [9,10].

Lithobiontic communities originate specific biodeterioration patterns (BPs), forming patinas, encrustations, pits, cracks, discolorations and detachments, which change in relation to lighting conditions and humidity values, and correspond to specific weathering patterns [11,12]. Studies on the rocks of the Negev desert (Israel) showed differentiable weathering patterns, which were explained by the effects of different environmental exposures, and related to different BPs [13]. This information was used for

interpreting BPs occurring in several archaeological areas in Israel and demonstrating the existence of different bioclimatic conditions before their burial, which stopped their growths but left their evident tracks [14,15]. In the case of the Trajan column (Rome), a diffuse pitting is still detectable on South-exposed marble surfaces, which had never shown any evidence of living organisms [16,17]. Such weathering patterns were interpreted as the consequence of past growths of endolithic communities of cyanobacteria and lichens, occurring in the areas wetted by incident rainfall [16]. The disappearance of such living communities, whose presence was recognisable in the historical photographs by typical blackening, was related to the building of the protective antibomb walls, during World War II, which caused a six-year period of darkness and the death of the photosynthesising microflora [16]. At present, the micro- and macroclimatic conditions of the area appear to be less suitable for the microflora in comparison to the past, and the chemical effects of dissolution are prevailing. Changes in biodeterioration phenomena on stone monuments in Rome were also related to the climatic changes between the 18th and 20th century, crossing evidence from past iconographic documentation and bioclimatic datasets [17–20]. Even if the communities are no longer living, due to the climatic changes that have occurred or peculiar environmental conditions in the past, the detected weathering patterns were useful in the understanding of past phenomena [19,20], and they may also potentially provide information for the future conservation of stone materials.

Regarding lichen tracks, in particular, biodeterioration studies have shown the correlation between pitting phenomena and the past presence of endolithic species [21]. The occurrence of oxalate deposits was also often related to lichen-driven biomineralisation processes [22,23] even if other possible causes can occur. This was proven in the case of the Trajan Column, on which past organic protective treatments favoured fungal growths and chemical oxidation processes [24]. On the other hand, lichens are producers of diverse secondary metabolites (more than 1000), variously localised through the thallus layers depending on their ecophysiological role [25–27]. Many of them are recognised bioactive substances, having different types of properties: antimicrobial, antialgal, antifungal, larvicidal, and herbicidal [26,28]. Although their secretion has long been associated to an allelopathic function [29,30], and as a factor regulating the development of lichen communities [30–32], the effects of such processes on stone surfaces have been poorly characterised. Some secondary metabolites with acidic and chelating functions have been characterised as agents of mineral leaching [33,34], but no study has ever verified if their allelopathic activity in situ could explain the presence of some tracks of uncolonised surfaces, which are compatible with the shape and size of lichen thalli and continue to appear clean after years. This could support the hypothesis of the long-lasting allelopathic properties of these substances and their potential application as natural products to control colonisation and biodeterioration by other microorganisms, as cyanobacteria and black fungi, on stone monuments [35]. Such new products are welcomed to improve the environmental sustainability of products used in restoration, since traditional biocides are often unsafe and removed from the market because of their toxicity [36–38]. Moreover, their potential long-lasting effects may contribute to the economic sustainability of stone heritage conservation [37].

The *Caestia* Pyramid in Rome, which still shows different evidence of previous colonisations, including lichens [17], and peculiar inhibition areas, is an interesting site to test such a hypothesis. Moreover, the rich historical iconography (paintings, photographs) available for the site and in situ observations carried out in recent decades give proof of the rich biological colonisation which occurred in the past, as well as of the rural context of the surrounding areas along the centuries (Figure 1).

Figure 1. Photographic view of the *Caestia* Pyramid dated back to 1880–1890, when it was at the border of the built area of the city (Alinari Archives, Aurelian Walls, Rome, 1880–1890 ca., unknown author, LVQ-L-000308-0023).

The Pyramid is a large cemetery monument (36.40 m high, with a square base of 29.50 m), built between 18 and 12 B.C. in honour of the rich politician and merchant Gaius Caestius, member of the religious corporation Epulonum collegium. The shape imitates the style of the Egyptian sepulchral buildings, as a sign of the recent extension of the political power of Rome [39]. It was built using stones and mortars, cladded with high-quality marble blocks extracted from the ancient quarry of Luni (between La Spezia and Carrara, Italy), which explains the name of "White" Pyramid. This famous Lunense marble is mineralogically dominated by calcite, with very small amounts of muscovite and dolomite-type carbonates [40]. The monument has been restored several times over the centuries, sometimes substituting the most weathered marble blocks with new ones, as in the case of the extensive and documented restoration which occurred in 1663 [41]. In that period, and over subsequent centuries, the biodeterioration phenomena were extensive, with a relevant growth of higher plants (Figure 1). Further investigations and restoration activities were carried out on the monument during the last few decades, and a survey of biodeterioration phenomena was made in the 1990s, showing the biological composition—due to cyanobacteria, fungi and lichens—of the diffuse dark patinas of the wall surfaces [17,42]. In more recent years, a survey was made before the last restoration (carried out in 2013–2014). The marble was colonised by many kinds of organisms, among which microorganisms forming a grey/black crust and including coccoid and filamentous cyanobacteria (genus *Gloeocapsa*, *Chroococcus* and *Scytonema*), and green algae (often lichenised on the surface of the marble), which also displayed endolithic penetration [43]. During the last restoration, diverse cleaning treatments were applied on such bio-patinas [44].

The aims of this paper are: (i) to give evidence of BPs on the Pyramid; (ii) to give a hypothesis on possible aetiological agent of the tracks of uncolonised surfaces; and (iii) to lay the basis for the selection of promising natural substances with long-lasting activity to be employed for stone conservation.

2. Materials and Methods

2.1. Evidence of the Diffuse Colonisation and BPs on the Pyramid During the Previous Centuries and Lichen Tracks

Observations of the biological cover in different historical periods were conducted on photographic materials at a high resolution level, collected by several archives (Table 1) and dating back to the end of 19th century up to today. We analysed the morphology of the different BPs patterns recognisable on such documentation. Generally, a detailed taxonomic identification was not possible, but we carried out an interpretation of possible colonisers, at the level of main phyla and, sometimes, also genera, considering the direct observations performed on residual communities in the 1990s [17]. The visible morphological characters were also helpful in identifying microorganisms, as algae and cyanobacteria tend to develop dark patinas, easily detectable in black and white photographs when considering their distribution related to incident rainfall or water percolation [11,19,20]. On the other hand, lichens usually show the borders of the thalli as evident patches, and mosses have the shape of pulvines, as previously observed in other historical documents [19,20]. In particular, we supported the interpretation of the growth inhibition areas and the possible effects of past lichen presence with the observation of close photographs taken in the 1990s, which were only partially used in a previous paper related to biopitting phenomena [17]. We also carried out further observations in the 2000s, before the last biocide treatments carried out in 1993 and 2003 and then between 2013 and 2018.

Table 1. Sources of the photographic material.

Archive	Number of Records
Archivi Alinari	31
Archivio Fotografico Giuseppe Primoli	8
Biblioteca Nazionale Centrale di Roma	1
Bibliothèque Nationale de France	1
CeDOT—Centro di Documentazione e Osservazione del Territorio dell'Università degli Studi Roma Tre	1
Cimitero Acattolico di Roma	6
CROMA—Centro per lo Studio di Roma dell'Università degli Studi Roma Tre	13
Deutsches Archäologisches Institut	20
ICCD—Istituto Centrale per il Catalogo e la Documentazione del Ministero per i beni e le attività culturali e per il turismo	29

2.2. Hypothesis on the Aetiological Agent of the Weathering Patterns and Selection of Promising Bioactive Substances

In order to evaluate the lichen species which possibly occurred in the past on the marble blocks of the *Caestia* Pyramid, we used historical records on lichen colonisation on carbonate lithotypes in Latium archaeological sites surveyed in the 1980s [45]. We also checked older historical data of the 19th century [46,47] on the occurrence of lichens on the Colosseum (Rome) even if they only partially dealt with carbonate substrates (mostly artificial stones, as mortars), because they could provide information on diversity and autoecological traits of the lichen flora in Rome in the past centuries.

We evaluated the potential correlation of lichen species recognised as possible colonisers of the Pyramid with the surfaces now noncolonised by cyanobacteria. In particular, the proposal of a taxonomical identification of lichens responsible for the inhibitory phenomenon was carried out considering the following factors: the morphological traits (growth form, thallus continuity and size), the production of secondary metabolites and autoecology of lichen species [48–50], their potential distribution in Rome, taking into account the different climatic conditions of the area in the last century [18,19], and the direct support by available photographic materials dating to the 1990s.

Given the typical secondary metabolites of the lichen species hypothesised by the previous elaborations [50,51], we derived the most probable compounds secreted on the surface. In a parallel

work, we also spectroscopically tested the evidence of metabolite traces potentially responsible for the inhibitory activity of certain species, the occurrence of which we hypothesised [52].

Finally, the potentially promising bioactivity of such lichen compounds was derived by PubChem [53] and specialised literature.

3. Results and Discussion

3.1. Evidence of the Past Colonisation and BPs on the Pyramid, Including Lichens and Their Tracks

The analysis of the dataset of about one hundred documents testifies the diffuse colonisation of the Pyramid, with blackening already recognisable in the oldest photographic documentation of the 19th century until the first half of the 20th century. Even if, in polluted environments, blackening phenomena can arise from residues of fossil fuels combustion—as detected on the *Caestia* Pyramid [42]—most of the blackish patinas and crusts were of biological origin [17,43]. Indeed, black crusts due to pollution phenomena are often detected in those areas protected from the leaching effects of rainfall [1,19,54]. Oppositely, when the blackening is located on the blocks wetted by rainfall—as in the case of the Pyramid—it has a clear biological origin, because water tends to dissolve and remove pollutants, in parallel favouring the biological growth [17].

All the historical photographic images display the prolonged presence of various BPs, which decreased in the last few decades. The current decrease in rainfall and increase in xeric conditions in the city have already been highlighted in the literature [55,56]. These micro- and bioclimatic changes and the recent restoration activities explain the reduction of BPs in recent years. Here, we report the following past BPs, which are distinguishable on old photographic documentation, as exemplified in Figure 2:

- blackish biofilms (BB), usually arising from cyanobacterial and fungal colonisations;
- lichen crusts (LC), as circular elements of lighter colour inside wide blackish areas;
- weeds, shrubs and arboreal flora (WS), detectable by a more complex organisation, with evident stems and leaves (sometimes also flowers). In these areas, the presence of mosses' pulvines can also likely be expected.

All the exposures showed diffuse BPs with variations during the old documented periods, likely as a result of restoration activities. The growth of higher plants between the blocks was significant in the oldest centuries, while a diffuse biological blackening is still evident in more recent times, particularly in the first half of the 20th century.

Photographic material of the 1990s showed subcircular tracks—in most cases of approximate 5–10 cm diameter, but sometimes larger in size—which remained free from surrounding growths (Figure 3c) or, sometimes, displayed a growth of black bio-patinas in the central parts of clearer areas (Figure 3a,b).

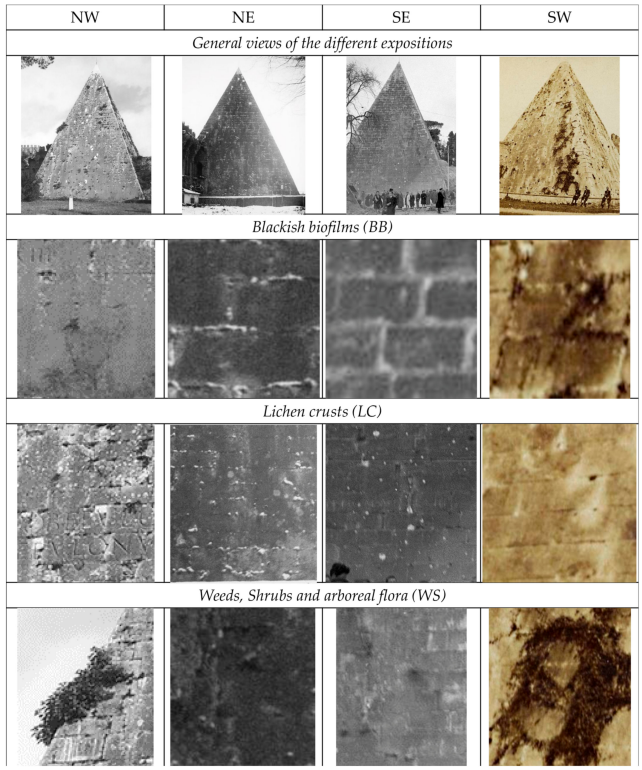

Figure 2. Selection of biodeterioration patterns (BPs) at different exposures from the end of 19th to the first half of 20th century: Northwest (NW)—1920–1930 (Alinari Archives, ACA-F-028699-0000); Northeast (NE)—1890 (Fondazione Primoli, 6639/A); Southeast (SE)—03–07/03/1944 (Alinari Archives, AIL-S-000659-0038); Southwest (SW)—1880–1890 (Alinari Archives, FVQ-F-042444-0000). The block height is approximately 50 cm.

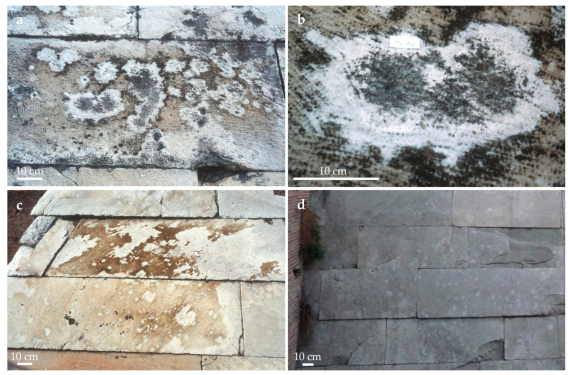

Figure 3. (**a**,**b**) BPs hypothetically related to previous lichen colonisation, and biofilm regrowth in their central areas (1990s); (**c**) BPs hypothetically related to lichen cover detachment (whitish surfaces) surrounded by brownish patinas (1990s); (**d**) BPs (whitish circular areas) hypothetically related to the past occurrence of lichen thalli, where the microbial recolonisation does not occur (2017). The block height is approximately 50 cm.

The morphology and size of such whitish areas seem compatible with the tracks of past lichen presence, which was no longer detectable on those blocks. In other portions of the walls, residues of some still living thalli of foliose cyanolichens (Figure 4a) and crustose chlorolichens were instead observed. These latter are potentially compatible with species of genera *Aspicilia* s.l. (Figure 4b) and *Verrucaria* s.l. (Figure 4c), and endolithic species responsible for pitting (Figure 4d).

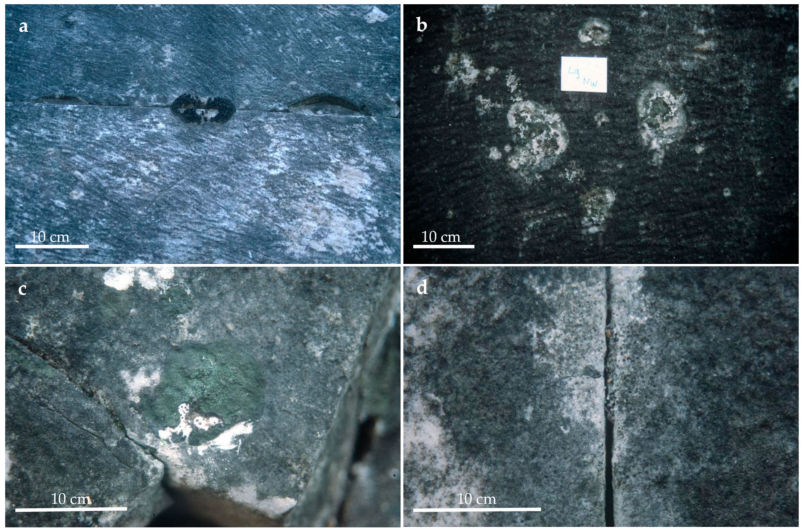

Figure 4. Lichens on the Pyramid before the restoration in the 1990s. Thallus morphology is potentially compatible with: (**a**) cyanolichens; (**b**) *Aspicilia* s.l.; (**c**) *Verrucaria* s.l.; and (**d**) endolithic species.

In recent photographs of 2017, tracks of lighter, uncolonised areas, which potentially delineate the shapes of past centimetric subcircular thalli, are still detectable on different exposures of the Pyramid (Figure 3d).

The decrease of biological colonisation in recent years can be explained taking into account several factors. In the first place, it is likely related to the increase in atmospheric pollution, considering its relevance in reducing biodiversity [53] and allowing only a few resistant organisms to survive when the microclimate is favourable [19]. Other negative factors were the above-mentioned reduction of humidity and rainfalls, as well as the increase in the temperature, which occurred in the last few decades, due to climatic changes and urban development [55,56]. Finally, restoration activities with traditional biocide treatments, which are carried out with higher frequency [44], contribute to maintaining the monument free from microbial colonisation.

3.2. Hypothesis on the Aetiological Agent of the Tracks

A lichen vegetation survey carried out during the 1980s through 16 archaeological sites of Latium displayed the total presence of 284 taxa [45]. Details on the distribution reported for each species clarify the peculiar association of 90 taxa with carbonate substrates, including travertine, hard and soft limestone, and marble. Figure 5 considers the morphological features and the production of secondary metabolites for these taxa, which may support or exclude their potential aetiological role in the development of the centimetric tracks of uncolonised surface (complete list of species in Supplementary Materials).

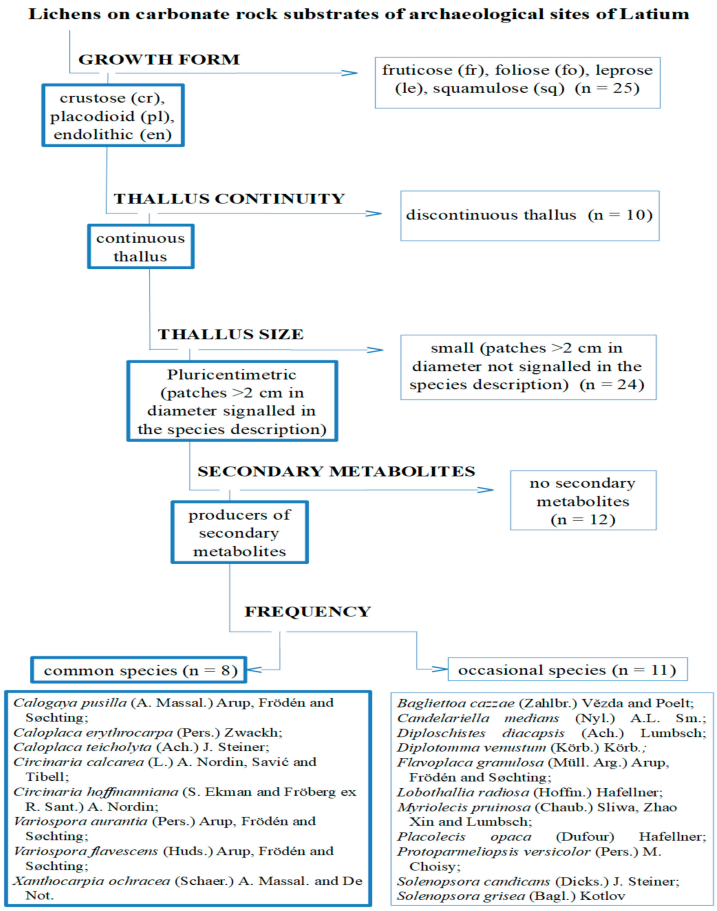

Figure 5. Lichens reported on carbonate rock substrates of archaeological sites of Latium [45], examined with respect to morphological and physiological traits potentially related with their recognition as aetiological factor for the development of the tracks of uncolonised surface (complete list of species in Supplementary Materials).

Sixty-five species display a growth-form compatible with a strict thallus adhesion and a deep interaction with the substrate, including epilithic crustose (n = 34) and crustose-placodioid (n = 12) species, and endolithic crustose ones (n = 19). Fruticose, foliose and leprose species (n = 25) generally show a limited and discontinuous contact with the rock substrate [4], which cannot be confidently associated with the continuity of uncolonised surfaces. Similarly, only 55 of the crustose species usually show continuous thalli, and only 31 of them generally show pluricentimetric thalli (patches > 2 cm), which may be compatible with the size of the tracks [45–47]. In this last group, twelve species do not produce any secondary metabolite, while the remnant 19 are known to produce secondary metabolites, at least in a part of vegetative or reproductive structures. On the basis of the reference vegetation survey [45], eight of these species are common on the carbonate stone surfaces, while an occasional occurrence characterises the other taxa.

Ecological indicator values [48] of all the 8 common taxa generally indicate a rather high to high xerophytism and tolerance for atmospheric pollution (Table 2), which are compatible with their presence in the area of Rome and the recent trend of climatic and atmospheric conditions. A strongly xerophytic and moderately nitrophytic lichen flora was also reported from Colosseum at the end of

the 20th century [46], while a higher number of hygrophytic species had been reported at the end of the 19th century [47].

Table 2. Lichen species hypothesised on the Caestia Pyramid and their ecological indicator values.

Species	pH	Solar Irradiation	Aridity	Eutrophication	Poleotolerance
Calogaya pusilla	3–5	4–5	4–5	2–3	1–3
Caloplaca erythrocarpa	4–5	4–5	4	2–3	1–2
Caloplaca teicholyta	3–4	3–4	3	3–4	2–3
Circinaria calcarea	4–5	3–5	4	2–3	1–2
Circinaria hoffmanniana	3–5	3–5	3–4	3–5	1–3
Variospora aurantia	4–5	4–5	4–5	3–4	1–3
Variospora flavescens	4–5	3–5	3–4	3–4	1–3
Xanthocarpia ochracea	5	2–4	3	1–2	1–3

Indicator scales according to Nimis [48] for: pH of the substrate: 3, on subacid to subneutral; 4, on slightly basic; 5, on basic; solar irradiation (s.i.): 2, in shaded situations; 3, in plenty of diffuse light but scarce direct s.i.; 4, in sun-exposition, but avoiding extreme s.i.; 5, very high direct s.i.; aridity: 3, mesophytic; 4, xerophytic, but not in extreme aridity; 5, very xerophytic; eutrophication (eu): 2, resistant to a very weak eu; 3, resistant to a weak eu; 4, in rather eu; 5, in highly eu; (v) poleotolerance: 1, in natural or semi-natural habitats; 2, in moderately disturbed areas; 3, in heavily disturbed areas.

In particular, six species of genera *Calogaya*, *Caloplaca*, *Variospora* and *Xanthocarpia* (grouped in the former genus *Caloplaca* s.l. before the revision by Arup et al. [57])—all producing antraquinones, including parietin ($C_{16}H_{12}O_5$, Figure 6a)—and two species of genus *Circinaria* (belonging to genus *Aspicilia* s.l. before the revision by Nordin et al. [58])—producing the polydroxylate macrolide aspicilin ($C_{18}H_{32}O_5$, Figure 6b)—were listed. It is worth noting that both *Circinaria* species also secrete oxalic acid, producing oxalate deposits within the thallus and at the rock interface [22,23,59].

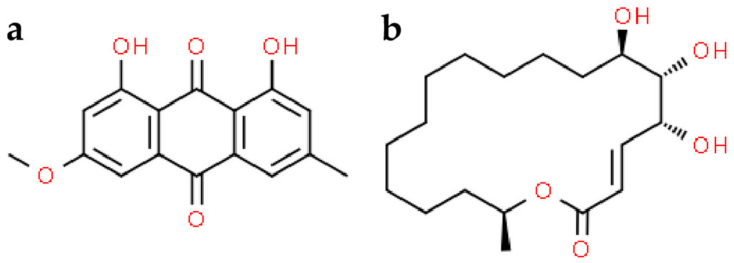

Figure 6. Chemical structure of parietin (**a**) and aspicilin (**b**).

With reference to the ecological indicator values, *Variospora* species, *Calogaya pusilla* and *Circinaria hoffmaniana* are more remarkably tolerant of strong aridity, direct solar irradiation, very high eutrophication and air pollution, likely associated to the microenvironment of the Pyramid walls. Nevertheless, only thalli compatible with *Aspicilia* s.l. were observed in the photographic material, while the presence of thalli of the *Caloplaca* s.l. group was never documented. Remarkably, *Variospora flavescens* and *Circinaria calcarea* were also observed in the 1990s on mortars in the Colosseum [46], and the latter species was already reported in the first floristic list dating to the end of the 19th century [47].

The eleven occasional species include producers of a wider set of secondary metabolites, such as depsidones (norstictic acid, psoromic acid, unidentified), depsides (lecanoric acid, diploschistesic acid), furandiones (usnic acid), pulvinic acid derivatives, xanthones (arthotelin, 2.7–dichloronorlichexanthone), terpenoids (zeorin) and aliphatic compounds (murolic acid).

The probable long-lasting growth-inhibition efficacy of lichens is supported by the known allelopathic activity of some of their secondary metabolites (see Section 3.3 below) and the characteristics

of the tracks. Moreover, spectroscopic investigations have been performed in parallel on carbonate heritage surfaces in Italy, others than the Pyramid, but analogously characterised by circular centimetric areas unaffected by the surrounding development of black biofilms [52]. In that case, thalli of *Circinaria calcarea* were still abundant nearby, and UV observations and Raman spectra compatible with aspicilin were obtained in the field and from material scraped from the uncolonised tracks, respectively.

On the Pyramid, the inhibitory effects—which are more evident on the borders of some tracks—can be explained considering the higher and more recent metabolic activity in the younger peripheral parts of thalli [60]. A complementary hypothesis is that the previous surface colonisation and the related hyphal penetration and chemical deterioration (e.g., by oxalic acid) may have predominantly modified the stone substrate and increased its bioreceptivity in the parts covered by the oldest parts of the thallus [61], prevailing on the inhibitory effect. However, this phenomenon is not generalisable, because in the centre of some tracks, we do not detect regrowth of bio-patinas (Figure 3c). For this reason, a difference in physical interactions does not thus seem sufficient to explain the differential growth inside and outside the circular tracks, whereas chemical differences due to different secondary metabolites produced by different lichens appear more likely to be aetiological agents.

3.3. The selection of Promising Substances

Biological activities of the secondary metabolites produced by lichen species reported on carbonate rock surfaces of Latium archaeological areas and having thalli compatible with the uncolonised tracks observed on the Pyramid are listed in Table 3.

Table 3. Biological activities of the secondary metabolites produced by the lichen species recognised as possible past colonisers of the Pyramid.

Lichen Compounds			Candidate Coloniser(s) of the Pyramid	Biological Activity	Reference
Category	**Metabolite**	**Formula**			
Polydroxylated macrolide	Aspicilin	$C_{18}H_{32}O_5$	*Circinaria calcarea*, *Circinaria hoffmaniana*	* Antibacterial activity, and phytotoxicity (inhibition of cholesterol biosynthesis and microfilament formation, antimalarial)	[62]
Antraquinones	Parietin (e.g.)	$C_{16}H_{12}O_5$	*Calogaya pusilla*, *Caloplaca erythrocarpa*, *Caloplaca teicholyta*, *Flavoplaca granulosa*, *Placolecis opaca* (medulla), *Variospora aurantia*, *Variospora flavescens*, *Xanthocarpia ochracea*	** antibacterial agent, antifungal agent, (apoptosis inducer, antineoplastic agent, hepatoprotective agent, anti-inflammatory agent)	[63]
Depsidones	Norstictic acid	$C_{18}H_{12}O_9$	*Diplotomma venustum*	* antimicrobial (antioxidant, anticancer)	[64]
	Psoromic acid	$C_{18}H_{14}O_8$	*Protoparmeliopsis versicolor* (medulla)	* antifungal activity (apoptotic activity)	[65,66]
Depsides	Lecanoric acid	$C_{16}H_{14}O_7$	*Diploschistes diacapsis*	* antibacterial, and antifungal (antitumor, antioxidant)	[67]
	Diploschistesic acid	$C_{16}H_{14}O_8$	*Diploschistes diacapsis*	not reported (at the best of our knowledge)	-
Furandiones	Usnic acid	$C_{18}H_{16}O_7$	*Protoparmeliopsis versicolor* (cortex)	* antibacterial, antifungal (antiviral, antitumour, antioxidant, antipyretic, analgetic, anti-inflammatory, hepatotoxic, antiviral)	[63,68]
Pulvinic acid derivatives	Calycin (e.g.)	$C_{18}H_{10}O_5$	*Candelariella medians*	** (antioxidant, photoprotection)	[69]
Xanthones	Arthothelin	$C_{14}H_7C_{l3}O_5$	*Myriolecis pruinosa*	** antimicrobial (antioxidant, cytotoxic)	[70]
	2.7-dichloronorlichexanthone	$C_{14}H_8C_{l2}O_5$	*Myrolecis pruinosa*		

Table 3. *Cont.*

Lichen Compounds			Candidate Coloniser(s) of the Pyramid	Biological Activity	Reference
Category	Metabolite	Formula			
Terpenoids	Zeorin	$C_{30}H_{52}O_2$	*Protoparmeliopsis versicolor* (medulla)	* antibacterial, antifungal (antioxidant)	[64]
Aliphatic compounds	Murolic acid	$C_{21}H_{36}O_5$	*Protoparmeliopsis versicolor* (medulla)	not reported (at the best of our knowledge)	-

* Biological properties reported for the metabolite category; ** Biological properties reported for the specific metabolite. Parietin, the antraquinone produced by all the *Caloplaca* s.l. species, is a well-known sun-screening compound [71], which protects thalli from excessive radiation, but it already revealed several additional potencies, including antibacterial and antifungal activities [63].

Aspicilin, produced by *Circinaria* species, has long been considered an attractive target to test synthetic methodologies, but its biological functions are still unknown. However, antibacterial activity and phytotoxicity were generally recognised for polyhydroxylated macrolides [62].

Antimicrobial functions, including antibacterial and antifungal ones, are known also for several secondary metabolites of the other species putatively related to the Pyramid tracks on the basis of distribution and morphological features, as for norstictic and psoromic acids (depsidones), lecanoric acid (depside), zeorin (terpenoid) and usnic acid (furandione).

Such data appear to be of great interest for monument conservation, considering that significant efforts have been made so far in order to control and hinder biodeterioration, often by means of biocides [36–38]. Traditional biocides have been used for a long time, but their extensive or inappropriate use may have negative implications [36,37]. Safer biocides are needed, in particular to protect the health of the operators—who handle big amounts of chemicals—to enhance environmental sustainability, and to preserve the integrity of the materials [38]. Such allelopathic compounds, produced by the putative past colonisers of the Pyramid, and in particular the macrolide aspicilin produced together with oxalates by *Circinaria* species, could be the source of new active principles to be tested for stone conservation. According to the recent proposals dealing with natural biocides [38], even very low doses of lichen metabolites seem to be valuable candidates as treatments to extend the efficacy of stone cleaning interventions [35]. Their natural long-lasting activity appears to be a further crucial element of economical sustainability in stone restoration. However, tests on their efficacy range and on the actual absence of interaction with materials or toxic effects on human health, poorly tested for such compounds, will be needed before their introduction in the field.

4. Conclusions

The observations and analyses of the rich archives of historical photographs available for the *Caestia* Pyramid in Rome suggested a lichen origin for the peculiar tracks characterised by evident inhibition areas for the growth of new bio-patinas. A precise evaluation of the changes over time in the cover of each BP will be the aim of a further contribution on the historical analysis of the monument. Considering the morphology, the biochemical properties and the historical data on lichens, occurring in Latium archaeological sites and monuments in 1980s and 1990s, we suppose that the tracks resulting from the secretion of allelopathic compounds by lichens mainly belong to the genus *Circinaria* (*Aspicilia* s.l.).

These results seem relevant to highlight the long-lasting allopathic properties of some lichen substances and to suggest further tests for an evaluation of their potential application as natural products to reduce stone monument colonisation. Considering their origin and activity, the environmental and economic sustainability of such compounds in the field of stone restoration seems promising.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/3/1132/s1, Complete list of lichens reported on carbonate rock substrates of archaeological sites of Latium [45], examined with respect to morphological and physiological traits potentially related with their recognition as aetiological factor for the development of the tracks of uncolonised surface.

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Diversity and structure of soil fungal communities used to unveil the past building history of a burial mound of ancient Japan (Tobiotsuka Kofun, Okayama Prefecture)
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Abstract: Kofuns are burial mounds of ancient Japan (3rd-7th century CE, defined Kofun Period). The construction history and archaeological significance of Kofuns of the Late Kofun Period (6th-7th century) still need to be characterized. This study dealt with Tobiotsuka Kofun (Okayama Prefecture, Japan) and aimed to investigate if diversity and structure of fungal communities, and their relationships with soil characteristics, beyond reflecting present vegetation, may be informative on the construction history of the burial mound, with particular reference to a supposed boundary between the original hill and the potential anthropic backfill. Soil fungal communities were thoroughly characterized by high-throughput sequencing (Illumina MiSeq technology) and metabarcoding, both at the ground and through different archaeological soil layers. Approx. 1.5 10^6 high quality ITS2 sequences, clustered in 1875 OTUs, were obtained from 59 sampling points, distributed inside the burial chamber and on the kofun external slopes. Heterogeneity and vertical distribution of fungal communities, considered in terms of taxonomic and trophic groups, generally showed a compatible pattern with present-days topographic conditions and vegetation. However, considering together the chemical soil analyses and fungal distribution with depth, in particular that of saprotrophic fungi, the data also point towards the presence of a former topsoil layer that was disturbed and at least partially buried.

Keywords: soil microbial communities, metabarcoding, microbial archaeology, next generation sequencing, trophic groups

Introduction

The diversity and structure of microbial communities are driven by abiotic and biotic factors, including climate, edaphic soil features, topography, vegetation, and human disturbance, of which they reflect present-day conditions (Gams, 1992; Kim et al., 2010; Tedersoo et al., 2014; Schlatter et al., 2018). Microbiological investigations on archaeological sites highlighted that the microbiota of anthropogenic soil layers can also bear the record of the activities of ancient people (Demkina et al., 2010; Peters et al., 2014, with refs. therein). Food waste disposal, fireplaces, the presence of animals, grain pits, manuring practices, dating centuries or even millennia, still leave different microbial tracks in archaeological layers (Peters et al., 2014; Ivanova and Marfenina, 2015; Chernysheva et al., 2017). The composition of present-day microbial communities might thus help in tracing the type and properties of organic matter in the soil even millennia after its deposition (Kim et al., 2010; Margesin et al., 2017), in particular through specialized biodegraders (e.g. keratinophilous fungi in presence of keratin deposits; Peters et al., 2014). Such an influence of ancient practices and historical soil management on the detectable microbiota was early recognized in cold and dry steppe zones of Russia (Demkina et al., 2007, 2008), but later reported from different bioclimatic areas, ranging from the Mediterranean basin (Siles et al., 2018) to the Brazilian Amazon (Grossman et al., 2010). The possibility that bacterial and fungal community structures represents an informative record of the burial and building history in archaeological sites, tracing the original stratigraphy and/or the contribution of external materials, similarly appears worth to be considered, but -at the best of our knowledge- still represents an unexplored perspective.

Kofuns are burial mounds of ancient Japan (3^{rd}-7^{th} century CE, defined Kofun Period) showing different size, shape and location depending on social features and the date of construction (Tsude, 1987; Knoph et al., 2018). In particular, the use of a stone chamber with a corridor entrance was introduced from Korea from the late 4^{th} century and became predominant during the Late Kofun Period (6^{th}-7^{th} century). However, such later burial mounds have been poorly investigated and still need to be characterized in terms of construction history and archaeological significance. The Tobiotsuka Kofun (Okayama Prefecture, Japan), dated to approximately late 6^{th}- early 7^{th} century CE, is one of these later burial mounds. Recent geophysical surveys characterized its stone chamber structure, the potential presence of a stone path and other construction features (Comina et al., 2020). However, the hypothesized boundary between the original hill on which the stone blocks delineating the chamber were positioned and (backfill) soil material, potentially put in place to cover the stone chamber and shape the mound, still needs to be clarified. A discontinuity revealed by geophysical analyses, at depths ranging from more than 1 m at the mound top to 0.2-0.3 m at its footslope, may indeed define the boundary, but it may also reflect different water contents and abundance of roots (Comina et al., 2020).

The aim of the present study is to assess if soil fungal communities, thoroughly characterized by high-throughput sequencing (Illumina MiSeq technology) and metabarcoding, beyond reflecting present vegetation, may be informative on the construction history of the Tobiotsuka Kofun, with particular reference to the supposed boundary between the original hill and the potential anthropic backfill. More specifically, we tested the hypotheses that (a) fungal diversity differs in trophic and taxonomic profiles at the ground level and in archaeological layers, both in the burial chamber and on the external slopes of the Kofun, and that (b) relationships between soil properties and fungal communities through the archaeological layers on the slopes of the mound express discontinuities incompatible with the current site conditions only.

Materials and Methods

Study site and soil sampling design

The topography and geo-pedological context of the Tobiotsuka Kofun [UTM ED50 53S N3837447, E 388805; 35 m a.s.l.; Fig. 1A] were recently summarized by Comina et al., (2020). In brief, the round shaped mound (diameter ~ 23 m; maximum height ~ 3 m) covers a stone chamber (approx. 13 m long, 1.5-2.3 m wide and 1.2-2.4 m high) walled and roofed by metric blocks of local magmatic stones, accessible from a hole (approx. 1 m wide and 1 m high) on the eastern slope which is opened since an undefined, long time.
Soil samples were collected during an archaeological excavation campaign in August 2019 from the burial chamber (C; n = 33) and the external slopes of the mound (E; n = 26) to carry out fungal diversity investigations and evaluate soil characteristics.The number and distribution of samples from each sampling area, at the ground level and from different archaeological layers, was planned in a balanced way, but finally depended on the effective possibility to collect the samples during the excavation. In the case of samples from archaeological layers, in particular, the selection of sampling points depended on the possibility to collect soil aliquots shortly after each excavation progress, to avoid potential effects of prolonged microclimate shifts on the fungal community, and to minimize the disturbance to the ongoing archaeological profiling and the non-excavated parts. No samples could be collected out of the burial borders due to limits of the excavation permission.

In the burial chamber, 15 sampling points were distributed at the ground level (i.e. 1-3 cm depth) at five distances from the chamber entrance (CG1-CG5 from the innermost one to the entrance) in groups of three (at 15 cm from the left and right stone walls, and in the centre) (Fig. 1B). Moreover, 18 sampling points were established at different archaeological layers recognized on the basis of soil colour and texture during the excavation of three vertical trenches, at approx. 9 m (CTA), 11 m (CTB) and 12 m (CTC) from the entrance (Fig. 1C). In particular, six sampling points per trench profile were distributed in groups of two or three at the different archaeological layers recognized during the excavation and at different distances from the stone walls. The depths of the sampled archaeological layers ranged from 5 to 40 cm (Fig. 1D, Table S1).

Outdoor, eight sampling points were established at the ground level on the top and the slopes of the mound (EG1-EG8; Fig. 1E). Moreover, 18 sampling points were established at the archaeological layers recognized on the profiles of two trenches (ca. 7 × 1 m) excavated on the southern (ETA) and western (ETB) slopes of the mound (Fig. 1F, Table 1). The sampling points were placed along the trenches at the backslope and footslope positions along the mound slope. The depths of the sampled archaeological layers ranged from 10 to 80 cm.

Soil samples from the ground level were collected with sterile spoons after the removal of the uppermost 2-3 cm layer of undecomposed and fragmented leaf and branch litter. Soil samples from the archaeological layers were collected using sterilized tubes, perpendicularly inserted at different depths of the trench profiles. After the collection, samples were processed in the laboratory to separate aliquots for soil and molecular analyses and stored at -20°C.

Soil analyses

The analyses were performed on the samples taken on the mound slope trenches and on those taken at ground level in the burial chamber. Soil samples were air dried and sieved at 2 mm for analysis. The C and N contents were determined with an elemental analyzer (CE 2100 Protein, Rodano, Italy). On the samples taken from the trenches, the total P contents were also determined after H_2SO_4–$HClO_4$ digestion (Kuo, 1996), with P detection by malachite green (Ohno and Zibilske, 1991).

Plant vegetation survey

Plant vegetation on the mound was surveyed in February 2019 in five 5×5 m^2 plots, divided into a grid of 25 quadrats, distributed on the top and the slopes (EP1-EP5; Fig. 1E). The frequency of plant species within each plot was calculated as the sum of their occurrences within the grid quadrats, evaluated at the ground. The relative importance of components of γ-diversity [i.e. similarity (S), relativized richness difference (D), and relativized species replacement (R)] was evaluated for all combination of plots by analyzing the matrix of species presence/absence with SDR Simplex software (2001) using the Simplex method (SDR Simplex; Podani and Schmera, 2011). A Principal Coordinate Analysis (PCoA; Ter Braak and Šmilauer, 2002) was performed to ordinate relevés on the basis of species frequencies at the plot level.

DNA extraction, PCR amplification and amplicon sequencing

Prior to DNA extraction soil samples were sieved (2 mm) to remove fine roots and large organic debris. Three independent extractions from each soil sample were performed using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of DNA samples were assessed by spectrophotometry (ND-1000 Spectrophotometer NanoDropH; Thermo Scientific). The nuclear ribosomal ITS2 region was amplified from all DNA extracts by means of the tagged primers primer pair ITSf9-ITS4 (White et al., 1990; Ihrmark et al., 2012). Each DNA extract was amplified in three replicates. The first PCR was performed using 0.4U of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Courtaboeuf, France), 1× Phusion HF buffer, 0.5 µM of each primer (ITSf9 and ITS4), 0.2 mM of each dNTPs and 1 µl of genomic DNA (20 ng), in a final volume of 25 µl. The PCR conditions used were: 5 min at 95°C, 35 cycles of 30 s at 94°C, 45 s at 56°C and 1 min at 72°C, followed by 10 min at 72°C. PCR products were checked on 1% agarose gel, and the three replicates of each sample were pooled and purified using The Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions. After quantification with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), the purified PCR products were mixed in equimolar amounts to prepare sequencing libraries. Two different libraries were prepared, one from the soils collected at the ground level, outside and inside of the mound, and one from the samples collected from the trench profiles. The libraries were paired-end sequenced using the Illumina MiSeq technology (2×300bp) by IGA Technology (Udine).

Bioinformatic analyses

Paired-end reads from each library were initially merged using PEAR v0.9.10 (Zhang et al., 2014), with the quality score threshold for trimming the low quality part of a read set at 28 and the minimum length of reads after trimming set at 200 bp. Assembled reads were then processed using Quantitative Insights into Microbial Ecology (QIIME) v. 1.8 (Caporaso et al., 2010). Initial sequence processing and sample assignment were performed with a minimum sequence length cut-off of 200 bp, minimum Phred quality score of 28, calculated over a sliding window of 50 bp, and allowing a maximum mismatch of 3 bp over the forward and reverse primers. Sequences were re-orientated when necessary to 5′ to 3′, and demultiplexed based on the tags and primers. Chimeric sequences were identified and removed performing a de novo (abundance based) detection using UCHIME (Edgar et al., 2011), as implemented in the QIIME pipeline. Operational taxonomic units (OTUs) were determined using an open reference-based clustering strategy, with the VSEARCH method, at 98% similarity; only clusters encompassing at least 5 sequences were retained. The UNITE database version 7.1 for QIIME was used as a reference for OTU picking and taxonomy assignment (https://unite.ut.ee/repository.php; last accessed February 2nd, 2019); BLAST algorithm (Altschul et al., 1990) was used as taxonomy assignment method, using 1e-5 e-value threshold. The OTU representative sequences generated in this study (i.e. the most abundant sequence within each OTU) were submitted to GenBank and recorded under the following string of accession numbers: MT594607 - MT596480.

Data analysis

A Venn diagram was constructed by means of the interactive Venn diagram viewer jvenn (Bardou et al., 2014). The trophisms of retrieved OTUs was defined by means of FUNGuild package (Nguyen et al., 2016). The two data sets, one containing the taxonomical assignation and the other the trophism assignation, each one composed of OTU table, taxonomy table and metadata, were then imported in Rstudio and were used to create 2 phyloseq objects with the R package phyloseq v. 1.22.3(McMurdie and Holmes, 2013). The two phyloseq object were than employed for the generation of Krona plots using Krona Tools and cpauvert/psadd library v.0.1.2 (Ondov et al., 2011). For different groups of samples (samples from ground level and trenches, from the burial chamber and the mound slopes), significant differences in the abundance of taxonomic and trophic groups was evaluated by means of ANOVA, with Bonferroni-corrected Mann Whitney test ($P<0.05$ as significant), using PAST (Hammer et al. 2001). The non rarefied OTU table was the used to carry out differential abundant analysis to identify OTUs that differ between the two sample categories investigated (samples coming from the surface sampling and from the archeological layer sampling). The differential abundant analysis was done by means of the R packages DESeq2 v 1.18.1 (Love et al., 2014), phyloseq v. 1.22.3 (McMurdie and Holmes, 2013), and ggplot2 v. 3.1.0 (Wickham, 2011). Relationships between fungal diversity and soil chemistry through the archaeological layers excavated on the mound slopes were analyzed using Canonical Correspondence Analyses (CCA, using biplot scaling for inter-species distances, Hill's scaling for inter-sample distances; choosing the forward selection of variables option; performing the Monte Carlo permutation test on the first and all ordination axes) were performed using CANOCO 4.5 (Ter Braak and Verdonschot, 1995).

Results

Soil analyses

The organic C contents in the soil taken at the ground level inside the kofun (CG1-CG5) showed a high variability (Table S1). The maximum contents (approx. 185-240 g kg^{-1}) were found in the set of samples taken on the left-hand side, but high contents (approx. 140 g kg^{-1}) were also visible in the right-hand side set. Remarkably lower amounts of C were visible closer to the kofun entry (approx. 25 g kg^{-1}). The N contents followed approximately the same trend, although some samples from the central set deviate, because of a high N contents with respect to C, likely because of some N contamination from bats. Excluding these data, the C/N ratio ranged from 8.6 to 19.6, a wide range of variation, which may be caused either by differences in the relative humidity, affecting microorganisms responsible of organic matter transformation or by the presence of organic matter originated from different species.

The samples taken at the ground level on the mound slopes (EG1-EG8) showed a C/N ratio (Table 1) which is in agreement with present-day vegetation, although some variations linked to site conditions were visible; some water stagnation close to the kofun entry, also suggested by vegetation analyses (see below) induced an increase in C/N up to values around 18, while the presence of lignin-rich residues is likely the responsible of the value of 20 found in EG6.

The chemical analysis of samples taken from the walls of the two trenches (Table 1), showed a general decrease of nutrients with depth, although in several cases, the decrease was irregular. In particular, the C and P contents, as well as C/N, increased again at 35-45 cm in trench A, both at backslope and footslope positions. On the walls of trench B, the trend was less marked and a high variability was visible among the samples taken at 10 cm depth.

Vegetation

A total of 30 plant species were recorded on the top and the slopes of the mound (Table S2). Broad-leaved evergreen (*Quercus glauca*) and deciduous (*Quercus variabilis*) Fagaceae, together with Ericaceae (*Vaccinium bracteatum*, *Lyonia ovalifolia*, *Rhododendron* sp.) and *Eurya japonica* (Pentaphyllaceae), displayed the highest frequencies (plot frequency 100%; quadrat frequency >15%) and dominated the tree and shrub layers of the forest vegetation. The SDR analysis (Fig. S1A) showed a remarkable species turnover between the plots (beta-diversity, R+D = 56%), and the PCoA (Fig. S1B) highlighted a higher association of the more hygrophilous vegetation component (Bryophyta and Cyperaceae) with the plot at the entrance of the kofun. Ericaceae are typical of acidic soils, while *Eurya japonica* and *Ardisia japonica* are indicators of a high level of soil disturbance (Manabe and Yamamoto, 1997; Nagamatsu and Miura, 1997).

Observations of the root systems emerging from the stone ceiling and the lateral stone walls of the burial chamber, some meters beneath the ground level at the top of the kofun, indicated a remarkable interaction of the tree and shrub vegetation with the burial environment of the heritage site.

Distribution of fungal diversity

After the bioinformatic analysis, 1,486,397 high quality ITS2 sequences (out of a total of 1,580,985 raw sequences) were retained and clustered in 1875 Operational Taxonomic Units (OTUs). A high number of sequences (n = 1,436,096), corresponding to 1844 OTUs, was retrieved in the soil samples from the ground level, while a two order of magnitude lower number (n = 50,301), corresponding to 266 OTUs, was retrieved from the archaeological layers recognized along the trench profiles inside the burial chamber and on the mound slopes.

With regard to the samples from the ground level, 1,422 OTUs (77%) were shared between the burial chamber (CG) and the external slopes (EG); 278 OTUs (15%) were specific of the interior, while 144 OTUs (8%) of the external slopes (Fig. 2). Most of OTUs (n = 266) retrieved in the trench profiles were also detected at the ground level (n = 235; 88%). However, 31 OTUs (12%) were exclusive of samples taken from the archaeological layers, and 96% of them (n = 30) were retrieved from both the chamber (CT) and external (ET) trenches.

Taxonomic and trophic fungal variability

In terms of taxonomic diversity (Table 2A; Fig. S2A), Ascomycota were mostly dominant in soil samples from the archaeological layers excavated within the stone chamber (CT, 71%), while their abundance was similarly lower (range 38-50%) in soil samples from the ground level (internal, CG, and external, EG) and the external trenches (ET). Basidiomycota were particularly abundant at the ground level, both inside and outside the chamber (38% and 30% respectively), while they occurred less frequently in the archaeological layers (approx. 10%). The presence of Mortierellomycota was remarkable outside the chamber, at the ground level (approx. 13%) and, particularly, in samples from the trench profiles (35%), while sharply lower within the chamber (< 4%). A significant occurrence of Mucoromycota was also detected at the ground level outside the chamber (6%), while their presence was lower in the other sample sets. It is worth noting that the percentage values reported for the different taxonomic groups in the different sample sets also depend on the number of unidentified taxa (ranging from 5 to 14%), which, however, should have a homogeneous impact on the whole dataset.

The ITS region sequence data were also used to infer the putative ecological roles of the total fungal communities at each sampled site. It was possible to assign a function to 1169 OTUs (62.3%) out of 1875 ITS2 OTUs. In terms of trophic diversity (Table 2B; Fig. S2B), the abundance of symbiotrophic fungi was higher at the ground level, both outside and inside the burial chamber, with respect to the archaeological layers. It was mostly due to ectomycorrhizal Basidiomycota (mostly Russulaceae and Cortinariaceae) and Ascomycota (e.g. *Cenococcum* sp.) and subordinate ericoid-mycorrhizal Ascomycota (e.g. *Oidiodendron* sp., *Meliniomyces* sp.) likely associated to the major groups of surveyed plants, as Fagaceae and Ericaceae. The saprotrophic component, instead, dominated in the archaeological layers (> 60%), particularly in the case of the mound slopes (> 70%), and was also conspicuous at the ground level (30-50%). Within the burial chamber, this dominance was mostly related to the presence of Ascomycota. On the mound slope the abundance of the saprotrophic-symbiotrophic, mostly endophytic, Mortierellomycota, both at the ground level

and within the archaeological layers, and of Mucoromycota, particularly at the ground level, was also remarkable (Fig. S2B).

Besides the differences in fungal communities recognized between the chamber and external areas, and at the ground level with respect to the archaeological layers, some heterogeneity was also detected through the different layers of the trench profiles (see paragraph below) and along the major axis of the stone chamber (Table S3). In this last case, in particular, the trophic distribution was rather homogeneous, but the abundance of the taxonomic groups changed, with Ascomycota and Basidiomycota relatively increasing and decreasing, respectively, from the entrance to the inner part of the chamber. Higher occurrence of animal pathogens at the bottom of the chamber was likely related to the presence of bats (CG1 13%; Table S3).

Fungal distribution and variability through the archaeological layers of the mound slopes

The above mentioned results indicated that the archeological layers are characterized by a specific fungal community that differs from that retrieved at ground level. Accordingly, the differential abundance analysis revealed that 26 OTUs out of the 31 were exclusive of the archeological trenches and other 21 (in common with the ground level) were significantly more abundant in the archeological layers ($P<=0.05$) (Fig. 3).

Patterns of variability in taxonomic and trophic diversity were detected through the archaeological layers of trenches ETA and ETB. When the abundance of different taxonomic (at phylum level) and trophic groups was considered in terms of number of sequences obtained, a remarkable variability between the different samples was a prominent feature, but some general trends were recognizable. In particular, Ascomycota and Mortierellomycota, i.e. the dominant phyla, increased and decreased respectively from lower to higher depths, with the exception of the sampling point at the footslope of ETB (ETB2F), showing an opposite trend (Fig. 4A). Taxa classified as strictly saprotrophic constantly increased with increasing depth, while saprotrophic-symbiotrophic and symbiotrophic decreased (out of ETB2F) (Fig. 4B). Some higher abundance of strictly pathotrophic fungi was detected at highest depths. When abundance patters were evaluated in terms of number of OTUs, a homogeneous distribution of taxonomic and trophic groups was generally observed (Fig. S3).

Such pattern is shown by the CCA of reads from ETA and ETB belonging in different trophic groups (CCA-I), which extracted four significant axes ($P < 0.05$), accounting for 100% of groups-environmental relationships (Fig. 5A). The first axis (76.9% of groups-environmental correlation) was positively related to depth, exhibiting the higher conditional factor according to forward selection ($F = 4.10$; $P < 0.05$), and negatively to soil P (1.84), C (0.8) and N (0.5) contents. Strictly pathotrophic and saprotrophic fungi were plotted in the right side of the diagram, positively related to depth, while symbiotrophic and symbiotrophic-saprotrophic ones were plotted in the left diagram, positively related to organic substance.

The CCA of OTUs (CCA-II) extracted four significant canonical axes ($P<0.05$) and showed an analogous distribution of depth and nutrients vectors (Fig. 5B). Depth ($F=2.11$) and soil N ($F=1.98$), were both significant conditional factors, being the first one positively related to the first axis (36.0% of s OTUs-environmental correlation), and the second more related to the second axis

(31.7%). Most of OTUs more differentially expressed with respect to the ground layer were positively related to depth and separately scattered in connection to distinct sampling points.

Discussion

Previous microbiological investigations on burial mounds of ancient Japan (late 7th-early 8th centuries) mostly focused on fungal and other microbial communities of stone chamber walls, responsible for biodeterioration of mural paintings and consequent conservation issues (Sugiyama et al., 2017). Our work on a more ancient burial mound (Kofun period), which does not show decorated interior walls, investigated soil fungal communities as potentially informative on its construction history and, particularly, on the hypothesized occurrence of (backfill) soil material put in place to shape the mound (Comina et al., 2020).

Most of the fungal diversity was associated with the surface soil, immediately beneath the litter of the warm temperate (secondary) forest surrounding the Kofun, with a two order higher OTUs richness with respect to the deeper layers exposed during the archaeological excavations. Strongly higher carbon contents (Tables 1 and S1), originated from litter or related to roots and their exudates (see Han et al., 2018 on *Q. glauca* forests), outclass the low C availability in the lower horizons (Fierer et al., 2003) and support a rich diversity of both symbiotrophs and saprotrophs, although the former are prominent (Carteron et al., 2020). Divergence between taxonomic and trophic structures of fungal communities at the surface of the burial chamber and the external slopes highlighted the expected impact of forest vegetation on fungal diversity. In particular, high abundance of Basidiomycota and symbiotrophic fungi at the surface of the stone chamber accounts for a dominance of ectomycorrhizal fungi (EcMF), in agreement with the observation of roots emerging from the walls and the *Quercus* tree vegetation on the mound top (Yamamoto et al., 2014; Toju et al., 2013). The higher occurrence of Mortierellomycota at the surface of the mound slopes likely reflects the current forest cutting practices to enhance site accessibility; wood harvesting might have favoured the increase of endophytic fungi (ENF) within decaying roots, with higher capabilities for degradation of complex organic compounds than EcMF (Kohout et al., 2018; Schlatter et al., 2018). Increasing trends in C and N from the entrance to the bottom of the stone chamber, and the associated shift from Basidiomycota to Ascomycota dominance, also accounted for the influence of site conditions on the fungal diversity at the surface. The more humid inner part of the chamber and the lower degree of anthropogenic disturbances with respect to the entrance may slow down organic matter decomposition thus preserving larger amounts of organic C in the soil. This hypothesis is supported by the lower C and N values found in the samples taken from the central area of the chamber, in particular with respect to the more humid left wall area (data not shown) The deep soil layers show different patterns of diversity and activity with respect to the dynamic topsoil, dominated by the tight coupling between vegetation and soil microbes (Engelhardt et al., 2018). Nevertheless, high abundance of the saprotrophic-symbiotrophic Mortierellomycota through the trench profile on the mound slope, compared to their poor occurrence in the archaeological layers of the stone chamber, signalled the influence of present-day vegetation, well beneath the ground layer, at least locally,

On the other hand, the fungal community in the archaeological layers significantly diverges from that of the topsoil in both quantitative and qualitative terms. This is in agreement with previous works showing that, in deep layers, microbial biomass decrease, as well as specialization and distinctness, are mainly driven by soil properties (Fritze et al., 2000; Du et al., 2017). Rather than in

terms of OTUs richness, different dominance of taxonomic and trophic groups is recognizable with reference to sequence abundances (Du et al., 2017; Li et al., 2020). In the case of the mound slopes, a vertical stratification was recognized, with the dominance of EcMF just beneath the surface (Schlatter et al., 2018; Carteron et al., 2020), vertically followed by a prominence of the mentioned saprotrophic-symbotrophic Mortierellomycota (Kohout et al., 2018), and finally by remarkable increases of saprotrophs at depths around 35-45 cm. Depth increase is the main driver of the community shift, inversely related to C, N and total P (CCA-I), but layers at 35-45 cm mostly show an increase of total reads with respect to related upper layers. A decrease in fungal abundance beneath the soil surface, followed by a new increase in deep layers is a known pattern when illuviation of C has occurred (Ekelund et al., 2001), but also occurs in buried A horizons of alluvial soils (Marfenina et al., 2009). Accordingly, such pattern of fungal distribution may agree with the countertrending C contents at approx. 40 cm of depth.

A high fungal diversity in deep soil layers was previously reported, in association with a higher variability with respect to upper layers, possibly associated to a greater heterogeneity in organic sources at the fine scale, the absence of the homogenizing effect of root growth, and minor dispersal (Kadowaki et al., 2013; Schlatter et al., 2018). Accordingly, the layers at depths of 35-45 cm and more hosted the OTUs more differentially expressed with respect to the surface layers (CCA-II). A remarkable presence of pathotrophs in deep layers is a reported phenomenon (Schlatter et al., 2018), as well as that of saprotrophic yeasts (as *Rhodotorula* and others), likely adapted to the oligotrophic conditions of deep soil layers (Li et al., 2020).

Based on these considerations, the heterogeneity and the vertical distribution of fungal communities generally showed a compatible pattern with present-days topographic conditions and vegetation. However, considering together the chemical soil analyses and fungal distribution with depth, in particular that of saprotrophic fungi, the data also point towards the presence of a former topsoil layer that was disturbed and at least partially buried. The C/N ratio in particular is similar to that of more superficial samples, and indicates poorly decomposed organic compounds at 35-45 cm; the depth trends in C and N also support the hypothesized disturbance event. The fungal communities at 35-45 cm would therefore reflect vegetation and organic matter before disturbances (Kim et al., 2010). Soil history was indeed shown to affect microbial communities, even after millennia and even more than the current land use (Grossman et al., 2010). The dominance of Ascomycota in the archaeological layers, and even in most of the ground layer samples from around the mound, is an additional hint in this direction. They indeed typically prevail in agricultural soils, including paddy soils (Li et al., 2020), while forest soils are usually dominated by Basidiomycota (Buée et al., 2009), whose occurrence is instead largely subordinate in the investigated site.

The dominance of Ascomycota indicates that only a partial equilibrium is reached between fungal communities and present-day secondary forests, but it might also point to an agricultural origin of backfilling soil materials used for mound shaping. Particularly in the case of deep soil horizons, microbial communities can long reflect the original depositional environment (Fierer et al., 2003), and this matches with an ancient A horizon covered during the mound building, although the current flourishing vegetation and the related symbiotrophic fungi superimpose and prevent the recognition of a univocal pattern.

In conclusion, diversity and structure of fungal community inhabiting the kofun soil, evaluated while taking into account also soil analyses, combine features compatible with the current site conditions and others which support an anthropogenic origin of a part of the soil.

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Tables

Table 1. List of soil samples from the external slopes of the mound (E; n = 26) and related chemical analyses.

Place (external, E; ground, G; trench A, TA; trench B, TB)		Sampling point	Mound zone (backslope, B; footslope, F; top, T)	Depth (cm)	Sample name	Sample symbol (not used, nu)	C ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	P ($mg\ kg^{-1}$)	C/N	C/P
E	G	1	F	2	EG1F2	nu	38.2	2.1	n.a.	18.2	n.a
E	G	2	F	2	EG2F2	nu	19.0	1.5	n.a	12.7	n.a
E	G	3	F	2	EG3F3	nu	23.9	1.9	n.a	12.6	n.a
E	G	4	B	2	EG4B2	nu	17.8	1.3	n.a	13.7	n.a
E	G	5	B	2	EG5B2	nu	19.6	1.7	n.a	11.5	n.a
E	G	6	F	2	EG6F2	nu	18.3	0.9	n.a	20.3	n.a
E	G	7	T	2	EG7T2	nu	13.7	1.0	n.a	13.7	n.a
E	G	8	F	2	EG8F2	nu	12.7	0.9	n.a	14.1	n.a
E	TA	1	B	25	ETA1B25	●	5.6	0.3	41.9	18.7	134
E	TA	1	B	45	ETA1B45	▼	6.2	0.3	43.0	20.7	144
E	TA	2	B	15	ETA2B15	●	7.2	0.6	51.2	12.0	141
E	TA	2	B	35	ETA2B35	▼	4.3	0.4	40.9	10.8	105
E	TA	3	B	10	ETA3B10	●	8.4	0.7	55.5	12.0	151
E	TA	3	B	30	ETA3B30	▼	4.2	0.5	27.7	8.4	152
E	TA	4	F	10	ETA4F10	●	9.2	0.9	57.2	10.2	161
E	TA	4	F	40	ETA4F40	▼	10.4	0.8	49.3	13.0	211
E	TA	4	F	60	ETA4F60	▼	3.3	0.4	37.2	8.3	89
E	TA	4	F	80	ETA4F80	▼	1.5	0.3	30.6	5.0	49
E	TB	1a	B	15	ETB1aB15	●	5.6	0.2	28.5	28.0	197
E	TB	1b	B	18	ETB1bB18	●	6.2	0.5	36.0	12.4	172
E	TB	1c	B	20	ETB1cB20	●	6.9	0.6	36.0	11.5	191
E	TB	1d	B	40	ETB1dB40	▼	2.3	0.1	33.4	23.0	69
E	TB	2a	F	10	ETB2aF10	●	7.6	0.7	45.0	10.9	169
E	TB	2b	F	10	ETB2bF10	●	7.7	0.3	40.5	25.7	190
E	TB	2c	F	10	ETB2cF10	●	12.0	0.9	57.1	13.3	210
E	TB	2d	F	40	ETB2dF40	▼	7.9	0.6	38.9	13.2	203

Table 2. Taxonomic distribution, at phylum level, and trophism (Sap, saprotrophic; Sym, symbiotrophic; Pat, pathotrophic) of OTUs retrieved from different sampling areas. Fungi showing multiple trophic behaviours are separately annotated). Data are expressed as percentage (mean ± SE). For each phylum and trophic group, values not sharing any letter are statistically different (One way ANOVA Mann-Whitney pairwise Bonferroni corrected P values; $P < 0.05$).

A) Taxonomy

Soil samples		Ascomycota	Basidio-mycota	Mortierello-mycota	Mucoro-mycota	unidentified
Stone chamber	Ground level (CG samples)	49.63 ± 4.11 b	37.74 ± 4.95 a	2.52 ± 0.88 c	2.03 ± 0.40 b	10.54 ± 2.40 a
	Archaeological layers (CTA-CTB-CTC samples)	70.69 ± 4.22 a	10.76 ± 4.65 b	3.70 ± 2.02 c	0.19 ± 0.07 c	14.61 ± 3.32 a
Mound slopes	Ground level (EG samples)	37.72 ± 5.98 b	30.23 ± 4.65 a	12.75 ± 1.20 b	5.02 ± 0.85 a	12.87 ± 3.72 a
	Archaeological layers (ETA-ETB samples)	46.80 ± 4.57 b	12.74 ± 2.65 b	34.83 ± 5.23 a	1.31 ± 0.18 b	4.24 ± 1.40 b

B) Trophism

Soil samples		Sap	Sap -Sym	Sap -Pat	Sap -Sym-Pat	Sym	Pat	Sym-Pat
Stone chamber	Ground level (CG samples)	28.12 ± 3.66 b	3.65 ± 1.09 b	0.6 ± 0.11 b	9.41 ± 3.61 a	52.16 ± 6.08 a	5.48 ± 1.42 b	0.58 ± 0.14 c
	Archaeological layers (CTA-CTB-CTC samples)	56.38 ± 6.81 a	4.68 ± 2.07 b	0.35 ± 0.06 b	8.04 ± 2.45 ab	20.24 ± 5.12 bc	5.37 ± 4.35 a	4.94 ± 1.95 b
Mound slopes	Ground level (EG samples)	27.73 ± 3.86 b	19.77 ± 2.21 a	4.48 ± 1.54 a	7.49 ± 2.19 ab	36.39 ± 7.46 ab	2.27 ± 0.44 b	1.86 ± 0.72 bc
	Archaeological layers (ETA-ETB samples)	29.74 ± 4.50 b	38.12 ± 5.59 a	5.01 ± 1.68 a	4.21 ± 1.65 b	7.79 ± 1.50 c	2.69 ± 1.10 a	12.44 ± 3.03 a

Figures

Fig. 1. Archaeological excavation at the Tobiotsuka Kofun and soil sampling sketches. A. NE-slope of the mound, showing the entrance of the burial chamber on the left side, with a stone block placed on top. B. Map of the burial chamber and distribution of sampling points at the ground level (green circles, CG) and in correspondence of trenches CTA-CTC (blue lines). C. Stone chamber. D. Excavation of trench CTA within the stone chamber. E. Topography of the burial mound and distribution of the vegetation relevés (orange squares, EP) and of soil sampling points at the ground level (green circles, EG) and in correspondence of the trenches on the southern and western slopes (ETA and ETB, respectively; blue lines). F. Trench on the southern slope during the excavation. Details on the codes of sampling points in Tables 1 and S1.

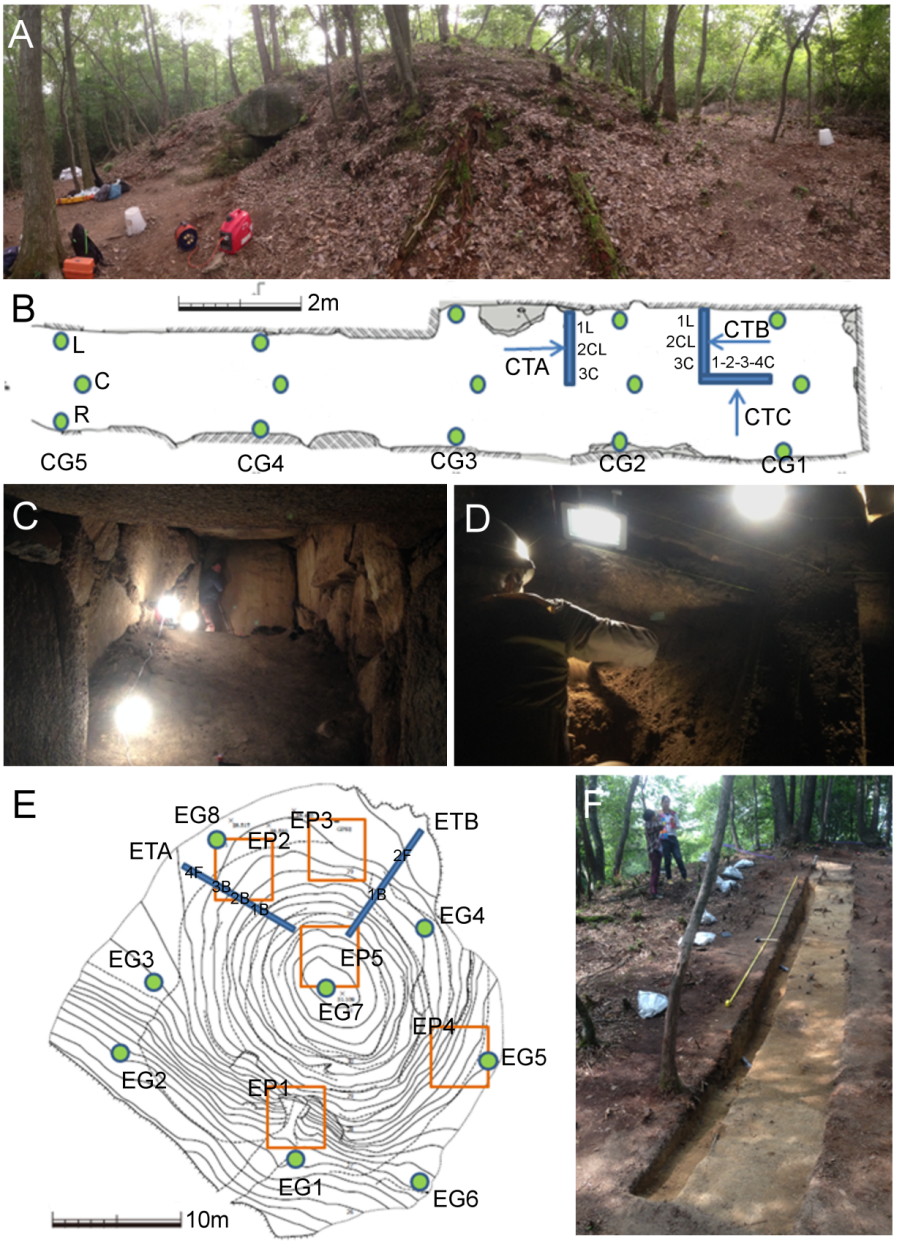


Fig. 2. Venn diagram showing the number of shared OTUs between samples from the stone chamber, at the ground level (CG) and from the trench profiles (CT = CTA+CTB+CTC), and from the external slopes of the Kofun, at the ground level (EG) and from the trench profiles (ET=ETA+ETB).

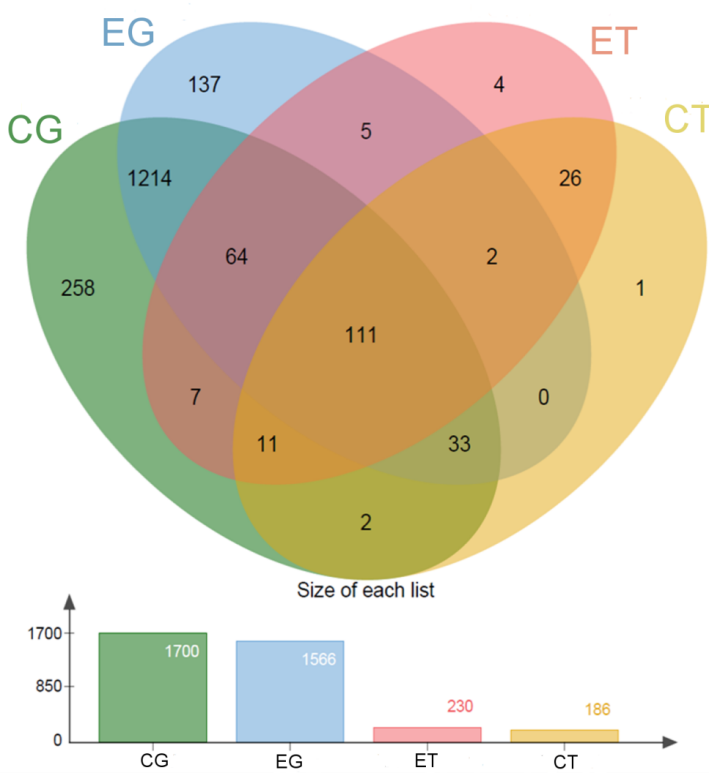


Fig. 3. Differential analysis of fungal OTUs whose abundance significantly differed at the ground level and the archaeological layers ($P < 0.05$). OTUs with a Log2FoldChange > 0 (on the left) were significantly more detected in the archaeological layers. The colours indicate the different fungal phyla (see legend).

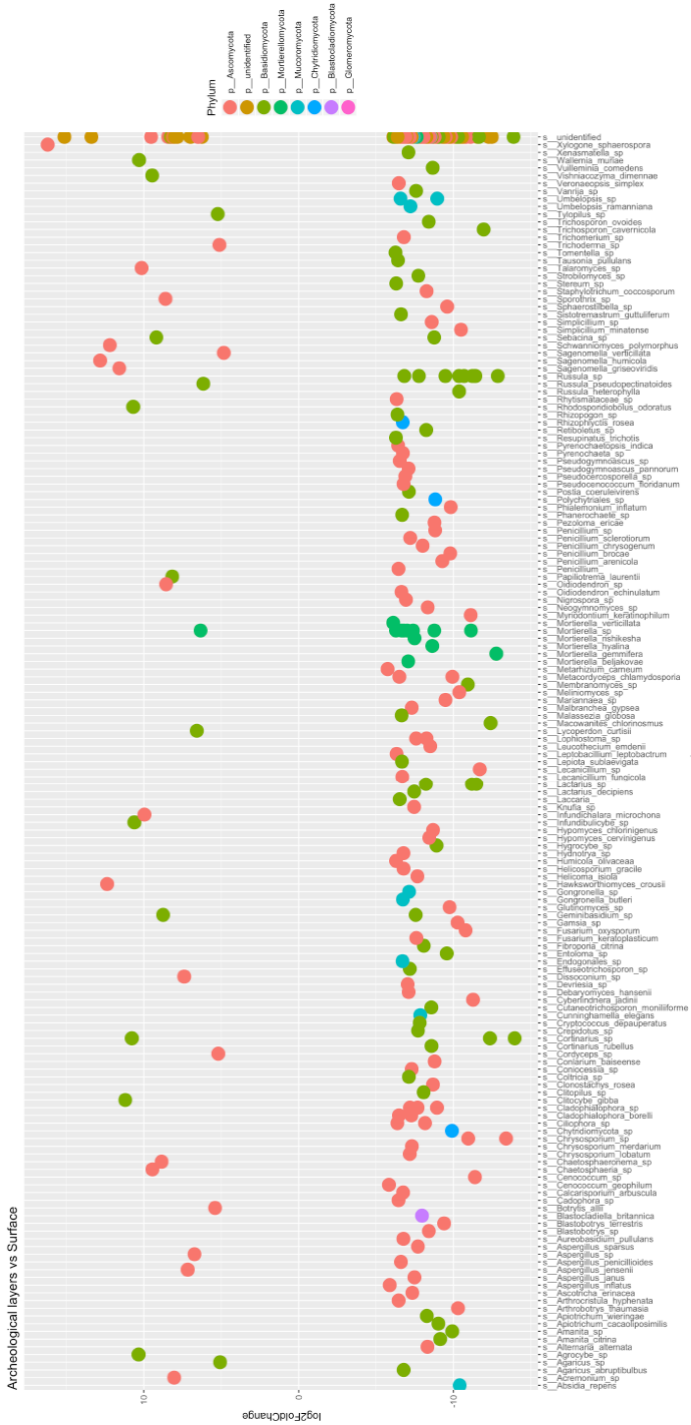


Fig. 4. Number of reads of different fungal phyla (A) and trophic groups (B; Pat, pathotrophic; Sym, symbiotrophic; Sap, saprotrophic; ND, not defined) at different sampling points through the archaeological layers of the burial mound.

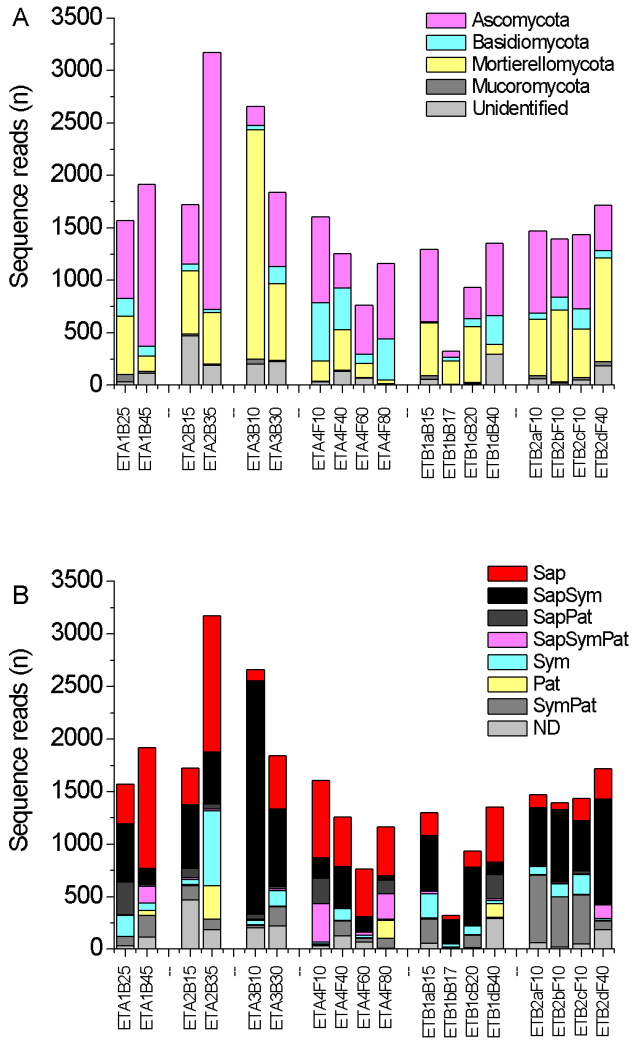
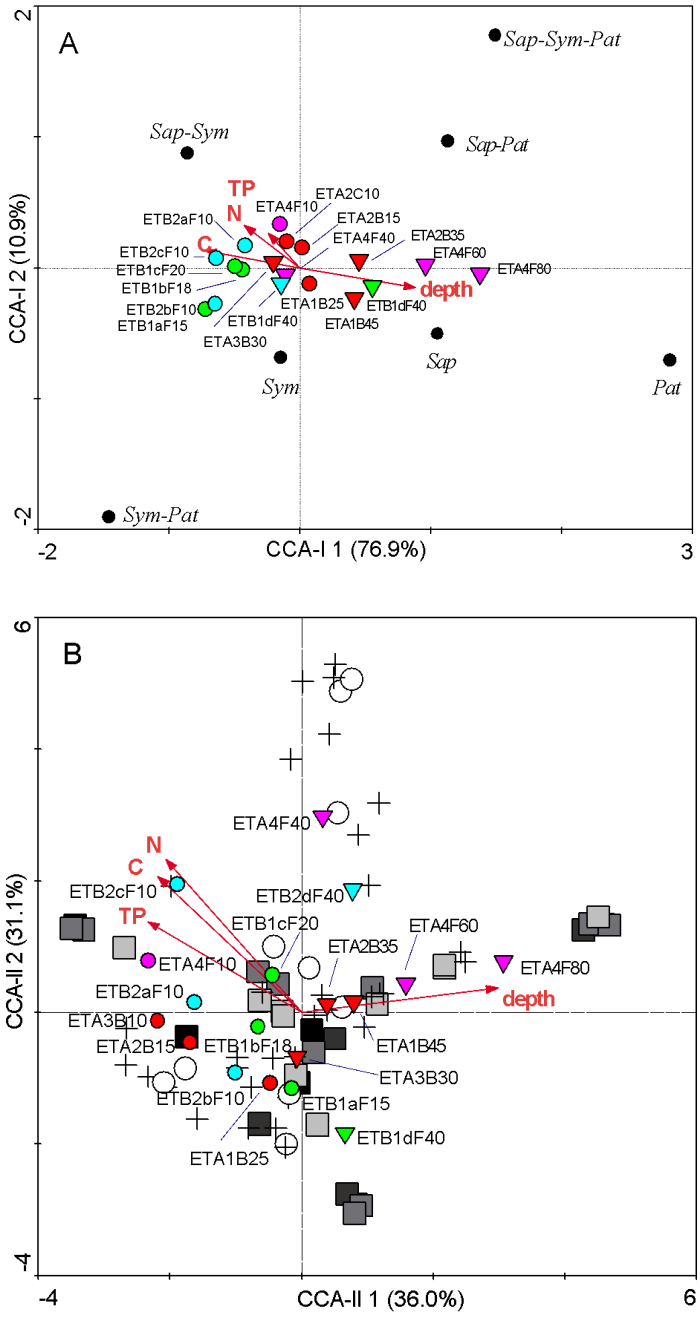


Fig. 5. Factorial maps in the canonical correspondence analysis (CCA) showing the position of trophic groups (CCA-I, A) and OTUs (CCA-II, B) with the contributions of soil factors (C, N, total P, and depth). Plot symbols indicate upper (circles) and deeper (down triangles) samples from different sampling points (different colours, as detailed in Table 1). In plot B, darker to lighter grey squares indicate OTUs more to less differentially expressed with respect to the ground layer; crosses indicate OTUs not differentially expressed with respect to the ground layer.



Supplementary materials

Table S1. List of soil samples from the burial chamber (C; n = 33) and related chemical analyses.

Place (chamber, C; ground, G; trench A, TA; trench B, TB; trench C, TC)		Sampling point	Distance from the entrance (m)	Side, looking from the entrance (left, L; central, C; right, R)	Depth (cm)	Sample name	C (g kg^{-1})	N (g kg^{-1})	C/N
C	G	1	11.5	L	2	CG1L2	239.2	24.9	9.6
C	G	1	12	C	2	CG1C2	44.1	12.3	3.6
C	G	1	11.5	R	2	CG1R2	142.8	15.6	9.2
C	G	2	9	L	2	CG2L2	186.5	21.6	8.6
C	G	2	9	C	2	CG2C2	12.5	12.2	1.0
C	G	2	9	R	2	CG2R2	97.0	9.0	10.8
C	G	3	6.5	L	2	CG3L2	98.0	10.3	9.5
C	G	3	7	C	2	CG3C2	49.1	4.6	10.7
C	G	3	6.5	R	2	CG3R2	146.3	14.8	9.9
C	G	4	3.5	L	2	CG4L2	77.6	6.9	11.2
C	G	4	4	C	2	CG4C2	35.2	2.9	12.1
C	G	4	3.5	R	2	CG4R2	31.0	2.8	11.1
C	G	5	0.5	L	2	CG5L2	25.9	2.0	13.0
C	G	5	1	C	2	CG5C2	81.4	4.2	19.4
C	G	5	0.5	R	2	CG5R2	25.2	1.9	13.3
C	TA	1	9	L	20	CTA1L20	n.a.	n.a.	n.a.
C	TA	1	9	L	35	CTA1L35	n.a.	n.a.	n.a.
C	TA	2	9	CL	15	CTA2CL15	n.a.	n.a.	n.a.
C	TA	2	9	CL	30	CTA2CL30	n.a.	n.a.	n.a.
C	TA	3	9	C	20	CTA3C20	n.a.	n.a.	n.a.
C	TA	3	9	C	35	CTA3C35	n.a.	n.a.	n.a.
C	TB	1	11	L	8	CTB1L8	n.a.	n.a.	n.a.
C	TB	1	11	L	35	CTB1L35	n.a.	n.a.	n.a.
C	TB	2	11	CL	12	CTB2CL12	n.a.	n.a.	n.a.
C	TB	2	11	CL	30	CTB2CL30	n.a.	n.a.	n.a.
C	TB	3	11	C	10	CTB3C10	n.a.	n.a.	n.a.
C	TB	3	11	C	25	CTB3C25	n.a.	n.a.	n.a.
C	TC	1	12	C	8	CTC1C8	n.a.	n.a.	n.a.
C	TC	2	12	C	10	CTC2C10	n.a.	n.a.	n.a.
C	TC	2	12	C	18	CTC2C18	n.a.	n.a.	n.a.
C	TC	3	12	C	8	CTC3C8	n.a.	n.a.	n.a.
C	TC	3	12	C	14	CTC3C14	n.a.	n.a.	n.a.
C	TC	4	12	C	8	CTC4C8	n.a.	n.a.	n.a.

Table S2. Plant diversity on the top and the slopes of the burial mound (data reported as frequency % through the five surveyed plots (EP1-EP5), and av. ± SD frequency % through the 25 quadrats surveyed per each plot).

Species	**Family**	**Abbrev.**	**Plot freq.**	**Quadrat freq.**
Bryophyta s.l.		Bry	100	64.8 ± 21.6
Thelypteris glanduligera (Kunze) Ching	Thelypteridaceae	Th.g	20	0.8 ± 1.8
Lepisorus thunbergianus (Kaulf.) Ching	Polypodiaceae	Le.t	40	2.4 ± 3.6
Dryopteris erythrosora (D.C.Eaton) Kuntze	Dryopteridaceae	Dr.e	20	0.8 ± 1.8
Juniperus rigida Siebold et Zucc.	Cupressaceae	Ju.r	80	7.2 ± 5.2
Cinnamomum camphora (L.) J.Presl	Lauraceae	Ci.c.	60	6.4 ± 7.3
Smilax china L.	Smilacaceae	Sm.c	80	4.8 ± 3.3
Ophiopogon japonicus (Thunb.) Ker Gawl	Asparagaceae	Op.j	20	0.8 ± 1.8
Carex conica Boott	Cyperaceae	Ca.c	40	12.0 ± 20.8
Bambusia sp.	Poaceae	Ba.s	80	16.0 ± 20.2
Cerasus jamasakura (Siebold ex Koidz.) H.Ohba	Rosaceae	Ce.j	40	1.6 ± 2.2
***Quercus glauca* Thunb.**	**Fagaceae**	**Qu.g**	**100**	**16.8 ± 8.2**
Quercus serrata Murray	Fagaceae	Qu.s	20	0.8 ± 1.8
Quercus variabilis Blume	Fagaceae	Qu.v	60	6.4 ± 6.1
Toxicodendron sp.[*T. succedaneum* (L.) Kuntze／*T. sylvestre* (Siebold et Zucc.) Kuntze／*T. trichocarpum* (Miq.) Kuntze]	Anacardiaceae	To.s	20	0.8 ± 1.8
Eurya japonica* Thunb. var. *japonica	**Pentaphylacaceae**	**Eu.j**	**100**	**80.0 ± 13.9**
Ardisia japonica (Thunb.) Blume	Primulaceae	Ar.j	60	19.2 ± 20.1
Ardisia crenata Sims	Primulaceae	Ar.c	40	3.2 ± 5.2
Lyonia ovalifolia (Wall.) Drude var. *elliptica* (Siebold et Zucc.) Hand.-Mazz.	Ericaceae	Ly.o	80	15.2 ± 13.4
Rhododendron serpyllifolium (A.Gray) Miq. var. *albiflorum* Makino	Ericaceae	Rh.s	60	11.2 ± 13.1
***Rhododendron* sp. (*R. macrosepalum* Maxim.／*R. reticulatum* D.Don ex G.Don)**	**Ericaceae**	**Rh.p**	**100**	**42.4 ± 36.2**
***Vaccinium bracteatum* Thunb.**	**Ericaceae**	**Va.b**	**100**	**20.8 ± 10.7**
Vaccinium oldhamii Miq.	Ericaceae	Va.o	20	1.6 ± 3.6
Gardenia jasminoides Ellis	Rubiaceae	Ga.j	20	1.6 ± 3.6
Trachelospermum asiaticum (Siebold et Zucc.) Nakai	Apocynaceae	Tr.a	20	1.6 ± 3.6
Ilex pedunculosa Miq.	Aquifoliaceae	Il.p	40	4.8 ± 8.7
Ilex crenata Thunb. var. *crenata*	Aquifoliaceae	Il.c	60	7.2 ± 7.2
Ilex chinensis Sims	Aquifoliaceae	Il.h	40	4.8 ± 8.7
Viburnum sp.	Viburnaceae	Vi.s	20	0.8 ± 1.8
Chengiopanax sciadophylloides (Franch. et Sav.) C.B.Shang et J.Y.Huang	Araliaceae	Ch.s	40	4.0 ± 6.9
Dendropanax trifidus (Thunb.) Makino ex H.Hara	Araliaceae	De.t	60	6.4 ± 6.7

Table S3. Taxonomic distribution, at phylum level, and trophism (SP, saprotrophic; Sym, symbiotrophic; Pat, pathotrophic) of OTUs retrieved from the ground level of the stone chamber at different distances from the entrance (CG samples). Data are expressed as percentage (mean ± SE). Fungi showing different trophic behaviours are separately annotated. For each phylum and trophic group, values not sharing any letter are statistically different (One way ANOVA Mann-Whitney pairwise Bonferroni corrected P values; P<0.05).

A) **Taxonomy**

Soil samples		**Asco-mycota**	**Basidio-mycota**	**Mortierello-mycota**	**Mucoro-mycota**	**unidentified**
Stone chamber	CG5 (entrance)	31.33 ± 10.07 a	53.31 ± 14.61 a	6.56 ±3.74a	2.32 ± 1.24 a	5.99 ± 2.48 a
	CG4	48.63 ± 10.84 a	43.50 ±10.13 a	2.24± 1.35 a	1.86 ± 0.66 a	3.33± 0.53 a
	CG3	49.27 ± 3.80 a	34.46 ±2.67 a	1.81 ± 0.75 a	1.06 ± 0.14 a	13.20 ± 5.63 a
	CG2	64.21 ± 6.25 a	15.75 ± 3.08 a	0.88 ±0.19 a	3.01± 1.34 a	14.76 ± 3.95 a
	CG1 (innermost part)	54.72 ± 5.73 a	26.69 ± 10.09 a	1.07 ± 0.40 a	1.80 ± 0.74 a	15.42 ± 6.33 a

B) **Trophism**

Soil samples		**Sap**	**Sap -Sym**	**Sap -Pat**	**Sap -Sym-Pat**	**Sym**	**Pat**	**Sym-Pat**
Stone chamber	CG5 (entrance)	22.18 ± 7.57 a	8.52 ± 4.66 a	1.12 ±0.31 a	12.65 ±9.46 a	51.02 ± 18.84 a	3.32 ± 1.27 a	1.19 ±0.26 a
	CG4	21.33 ± 0.96 a	2.91 ± 1.67 a	0.35 ± 0.08 a	20.13 ± 16.01 a	53.08 ± 16.84 a	1.54± 0.50 a	0.64 ± 0.44
	CG3	23.39 ± 3.25 a	2.73 ± 0.89 a	0.37 ± 0.04 a	3.34 ± 0.88 a	67.14 ± 5.17 a	2.62± 0.58 a	0.40 ± 0.29 a
	CG2	44.1 ± 13.05 a	2.45 ±0.69a	0.72± 0.36 a	6.73± 2.68 a	38.34 ± 17.13 a	7.35 ± 1.95 a	0.30 ±0.10 a
	CG1 (innermost part)	29.59 ± 7.05 a	1.65± 0.41 a	0.42 ±0.07 a	4.19 ± 2.08 a	51.22± 11.30 a	12.58 ±4.82 a	0.34 ± 0.16 a

Fig. S1. SDR simplex ternary plot (A) and PCoA (B) for the plant plots.

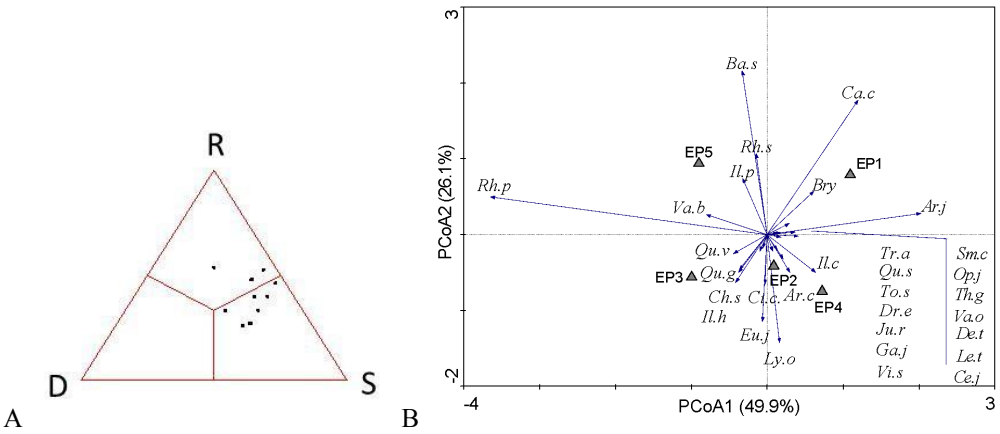
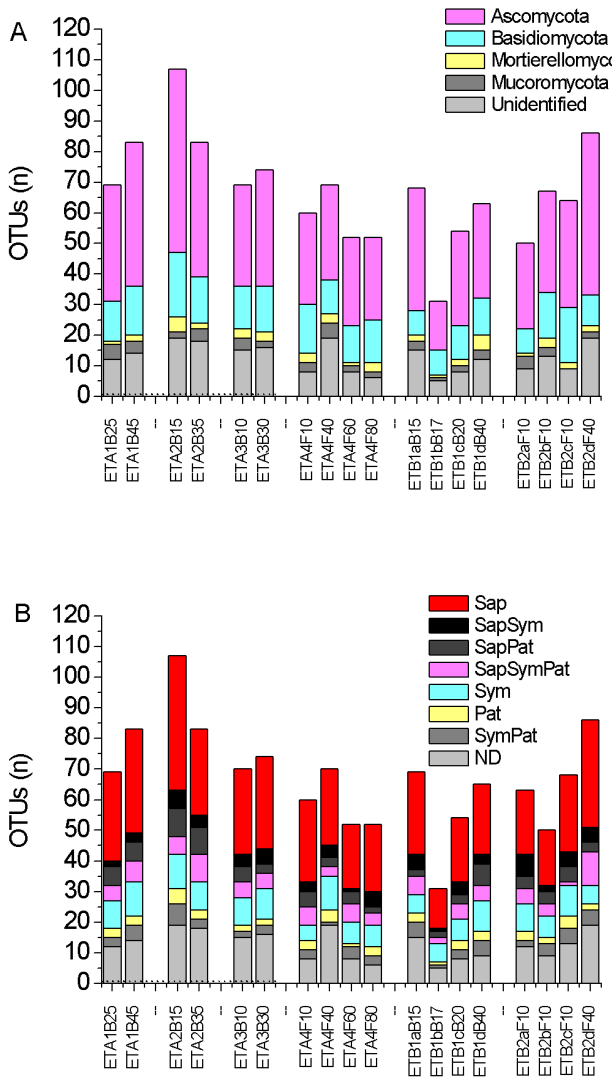


Fig. S2. Taxonomic (A) and trophic (B) kronas of detected fungal OTUs.

https://figshare.com/s/f43408a6d27fc1416045

Fig. S3. Number of OTUs of different fungal phyla and trophic groups (P, pathotrophic; Sy, symbiotrophic; Sa, saprotrophic; ND, not defined) at different sampling points through the archaeological layers of the burial mound.



Additional comment to Fig. S3 - A similar number of sequences (approx. 2000) corresponding to a similar number of OTUs (approx. 65) was retrieved in the two set of samples collected at layers 2 (10-25 cm) and 2-3 (30-45 cm) of ETA, respectively, in different parts of the mound backslope (sampling points 1B, 2B, 3B). Remarkably, the abundance (number of OTUs and %) of trophic groups was consistent for each layer through different parts of the mound slope (from the upper to the lower backslope), with saprotrophic-symbiotrophic (mostly *Mortierella* sp.) and saprotrophic (mostly *Sagenomella* spp.) dominating the upper and lower layers, respectively, and symbiotrophic increasing from the upper to the lower. At the footslope of the same trench ETA (sampling point 4F), at depths ranging from 10 to 80 cm, the retrieved sequences corresponded to a rather constant

number of identified OTUs (approx. 50), but the number decreased from 10 cm (1600) to 60 cm (700), and then increased at 80 cm (1153). Saprotrophic-symbiotrophic fungi (mostly *Mortierella* sp.) and symbiotrophic (ectomycorrhizal and endophytic) were more abundant at 40 cm with respect to 10 cm, and then gradually decreased at the two deeper layers. A high number of sequences of saprotrophs was detected at 10 cm (mainly *Aspergillus jemsenii, A. amstelodami, Wallemia muriae* and *Infundibulicybe* sp.) and they were percentually dominant at 60 cm (mainly *Sagenomella*). Most of "pathotrophic categories" were more abundant at 10 and 80 cm (Sap-Pat: *Chaetosphaeronema* sp. and *Clitocybe gibba*; Sap-Sym-Pat: *Vishniacozyma carnescens* and *Acremonium variecolor*) or 80 cm only (Pat: *Mycosphaerella*), while pathotrophic-symbiotrophic (mostly *Oidiodendron* sp.) were more abundant at 40, 60, 80 cm than at 10 cm.

With regard to trench ETB, sequences and OTUs retrieved from deep samples collected at 40 cm in sampling points 1d and 2d were similar (or slightly higher) than those retrieved from corresponding upper layers at 10-20 cm. At the backslope, as in the case of the other trench, saprotrophic-symbiotrophic (*Mortierella*) were more abundant at the upper layer and saprotrophic (*Aspergillus jensenii, Lycoperdon curtisii, Penicillium spinulosum, Leptobacillium leptobactrum*) at the deeper one, where the presence of pathotrophic (*Mycosphaerella*) and pathotrophic-saprotrophic (*Botrytis allii, Rhodosporidiobolus odoratus*) was also remarkable. At the footslope, as in the case of the other trench), a different pattern was observed, with saprotrophic-symbiotrophic (*Mortierella*) more abundant at the deeper layer, a remarkable abundance of pathotrophic-symbiotrophic (*Oidiodendron* sp.) at the upper layer, and a lower abundance of saprotrophs (*Sagenomella*).

Oral Communication by Chiara Tonon at the International Association for Lichenology 9th Symposium (IAL9, Virtual Symposium): "Unlocking the inner lichen" – 1-6 August 2021.

PROTEOMIC AND SPECTROSCOPIC ANALYSES OF *Bagliettoa baldensis* (A. MASSAL.) VĚZDA: INSIGTHS INTO THE ENDOLITHIC GROWTH OF LICHENS

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Almost one hundred years after EJ Fry's accurate picture of endolithic lichen growth within limestone (Ann. Bot. 36, 1922), the underlying physiological and biogeochemical processes are yet to be fully disentangled. Early hypotheses on a carbonic acid-driven calcite dissolution, related to CO_2 release by respiration, have already been supplemented by evidences of the mycobiont production of chelating metabolites and of carbonic anhydrase, which might modify dissolution and precipitation equilibria. In this study, proteomic and spectroscopic investigations on *Bagliettoa baldensis* (A. Massal.) Vězda from marble outcrops in Carrara (Italy) suggest further degrees of complexity. We extracted the acid-soluble protein fraction, which was analysed by high-resolution liquid chromatography-tandem mass spectrometry. This "shotgun" approach allowed us to identify hundreds of protein sequences, which encompass the different key functions of the symbionts, including photosynthesis, respiration, stress response, protein synthesis, and DNA structuring; remarkably, it also shows the presence of fungal proteins involved in the glycerolipid pathways, not reported in the profile of other lichens. Such finding is in agreement with the well-known fact that endolithic fungi grow more or less inflated "oil hyphae", which we also observed in *Bagliettoa* apomycobionts upon long-term culturing. We carried out µ-Raman analyses of the oil hyphae in vitro, which were compared to those of the colonized Carrara marble, obtained after strong bleaching with concentrated (12% w/v) sodium hypochlorite to remove surface-bound biomolecules. Spectra attributable to fatty acids and tryacylglycerols were collected both on the oil hyphae and the "clean" marble, also suggesting their incorporation within calcite crystals. In the wake of the pioneer questioning by EJ Fry on the involvement of oil hyphae in the endolithic growth pattern, a potential role of glycerol and glycerolipids in dissolution and biomineralization of calcite by *B. baldensis* will be discussed, also with reference to other biological systems.

Lightning Talk by Sergio E. Favero Longo at the International Association for Lichenology 9th Symposium (IAL9, Virtual Symposium): "Unlocking the inner lichen" – 1-6 August 2021.

LONG-LASTING EFFECTS OF ASPICILIOID LICHENS ON THE BIORECEPTIVITY OF CARBONATE SUBSTRATES

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Aspicilioid lichens have been indicated for the biodeterioration activity on stone cultural heritage, mostly because of secretion of oxalic acid and formation of calcium oxalates. However, the involvement of its pattern of metabolites in biogeochemical and other interactions on the carbonate substrates has not been fully clarified. In this study, we examined the still present *Circinaria* gr. *calcarea* and its past probable tracks occurring on marbles in the Garden of a 17th century Residence of the Royal House of Savoy (Villa della Regina, UNESCO-WHS 823bis, Torino, NW-Italy). The marble balustrades, after their restoration ended in early 2000s, display a widespread recolonization by a black biofilm of cyanobacteria and MCF, and a lichen community including *C. calcarea*, but they also remarkably show several clearly defined, centimetric, circular areas unaffected by the recolonization dynamics. We aimed to verify if these areas may be the tracks of the lichen colonization preceding the last restoration and, in particular, of *C. calcarea* thalli, resulting compatible in shape and size. Beneath scraped thalli of *C. calcarea*, X-ray diffraction patterns of calcite showed a remarkable stability of crystallographic planes which are known to be enhanced in presence of organic substance. The same pattern was recognized in correspondence of the uncolonized circular areas, but not where the black biofilm spread. UV observations (365 nm) also showed similar signals beneath the thalli and in the uncolonized areas. Moreover, Raman spectra obtained from methanol- and acetone-extracted thalli and the marble powder gently scraped from the uncolonized areas displayed similar patterns, compatible with lichen metabolites. Such findings suggest a durable effect of lichen colonization on the bioreceptivity of the marble. Both the entrapment of metabolites with allelopathic functions and changes in the rock microstructural properties due to biomineralization appear potential causative factors.

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