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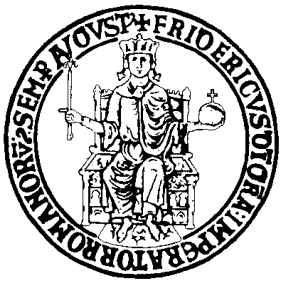

DOCTORATE SCHOOL IN BIOLOGY

Cycle XXX

Development of non-invasive diagnostic methods for monitoring biodeterioration of monuments

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Academic Year 2016 – 2017

INDEX

Synopsis

A large part of the world's most precious cultural heritage and artworks are made of stone with a finite life, and they are slowly but irreversibly disappearing. Biofilms living at rock-atmosphere interface are heterogeneous, complex consortia whose ability to alter properties of the substratum is defined *biodeterioration*. This phenomenon received serious attention by scientists only within the last three decades. The present work is aimed to advance the understanding of mechanisms involved in microbial biodeterioration: new investigation tools for the investigation of the biofilms are indeed required, so that the needs of small amount of sampling material to be analyzed in non-invasive and highly reproducible assay can be satisfied. Three sampling campaigns were carried out at the archaeological sites of Oplontis, Pompeii and at Phlegrean Phields.

In a series of *in vitro* colonization experiments, the pioneer attitude of the fungi *Fusarium solani* and *Alternaria tenuissima* as well as the cyanobaterium *Oculatella subterranea*, was tested and monitored for a short-term period. Through the use of many variants of microscopy included CLSM and computer image analysis it has been possible to depict fine structure and architecture of the studied microrganisms, in a controlled environment where the realistic conditions of the respective sampling points have been reproduced.

A novel approach for the study of subaerial biofilms via the construction of qPCR primers and fluorescent internal probes is also proposed, based on a deep survey on microrganisms occurring over stone monuments in European countries.

A further proposed tool is the characterization of microbial diversity through the use of flow cytometry; phototrophic components of sampled biofilms were analyzed with flow cytometry, which allowed the sorting of the two Genera *Cyanidium* and *Galdieria*. Species identification was later obtained with the use of novel-designed species-specific primers targeting plastidial gene rbcL.

Larga parte del patrimonio culturale e delle opere d'arte più preziose al mondo ha i giorni contati e sta lentamente ma irreversibilmente scomparendo. Le patine microbiche che vivono all'interfaccia roccia-atmosfera sono consociazioni complesse ed eterogenee la cui capacità di alterare il substrato è definita come biodeterioramento. Tale fenomeno ha ricevuto l'opportuna considerazione da parte degli scienziati solo negli ultimi trent'anni.

Il presente lavoro si propone di avanzare la comprensione dei meccanismi coinvolti nel biodeterioramento microbico: sono perciò necessari nuovi strumenti d'investigazione per lo studio delle patine microbiche, tali da richiedere piccole quantità di campione e che possano essere analizzate in saggi non invasivi ed altamente riproducibili. Sono state condotte tre campagne di campionamento nei siti archeologici di Oplonti e Pompei e ai Campi Flegrei.

In una serie di esperimenti di colonizzazione in vitro è stata monitorata a breve termine la capacità pioneristica per i funghi Fusarium solani *ed* Alternaria tenuissima*, così come per il cianobatterio* Oculatella subterranea*. Attraverso l'uso di molte varianti di microscopia, inclusa quella CLSM unita all'analisi digitale di immagini, è stato possibile rappresentare la struttura fine e l'architettura dei microrganismi studiati, in un ambiente controllato ove le condizioni dei rispettivi punti di campionamento sono state riprodotte realisticamente.*

Un nuovo approccio per lo studio delle patine microbiche è inoltre qui proposto mediante la costruzione di oligonucleotidi per qPCR così come l'utilizzo di sonde interne fluorescenti, basate su una approfondita ricerca dei microrganismi ritrovati sui monumenti in pietra in tutta Europa. Un ulteriore strumento proposto è la caratterizzazione della diversità microbica mediante citometria a flusso; le componenti fotoautotrofe delle patine campionate sono state analizzate in citofluorimetria, che ha permesso di isolare i due generi Cyanidium *e* Galdieria*. L'identificazione delle specie è stata poi ottenuta con l'ausilio di oligonucleotidi specie-specifici di nuova realizzazione costruiti sul gene plastidiale rbcL.*

CHAPTER 1
GENERAL INTRODUCTION

1. 1 Main features of subaerial biofilms

The term 'subaerial biofilm' (SAB) has been introduced for microbial communities that develop on solid mineral surfaces exposed to the atmosphere. These communities are ubiquitous and self-sufficient microbial ecosystems that may be found on buildings, monuments and bare rocks at all latitudes where direct contact with the atmosphere and solar radiation occurs (Gorbushina, 2007; Caneva et al, 2008).

These patinas are composed by densely packed microorganisms that live in self-organized structures of micron to millimeter scales. Made up of a moltitude of many different microbial cells, the exertion of coordinated survival strategies increases biocide resistance and microbial fitness, and avoids the loss of energy and nutrients (Stewart and Franklin 2008, Stone 2015). All major metabolic groups of microorganisms can be found, including chemolithotrophs, chemoorganotrophs and phototrophs (Gorbushina, 2007).

1.1.1 Ecology of subaerial biofilms

Terrestrial cyanobacteria and algae are pioneer organisms, which colonize habitats potentially unavailable for living organisms and transform them, giving the opportunity to other organisms to settle (Schopf et al., 1996). Tipically, phototrophic biocenosis may allow the later growth of more complex communities, including the heterotrophic microbiota (Tomaselli et al., 2000). The association of phototrophic components embedded in a biofilm enriches itself with organic and inorganic substances and growth factors (Tiano, 2002) providing an excellent nutrient base for the subsequent trophic succession. However, the establishment of heterotrophic communities on rocks is possible even without the pioneering participation of phototropic organisms and may in fact facilitate the subsequent growth of photosynthetic populations (Roeselers et al., 2007). In this case, organic substrates from various sources are used, including airborne particles and organic vapors, organic matter naturally present in sedimentary rock (usually between 0.2% and 2%),

excreted organic metabolic products and biomass from other organisms (Warscheid and Braams, 2000; Urzì, 2004).

Stone-atmosphere interface can be considered as an extreme environment characterized by severe environmental fluctuations. Especially desiccation, low nutrient concentrations, large temperature variations, high exposure to wind and UV radiation are some of the features of this stressful habitat (Viles and Cutler 2012). For this reason, only microorganisms with a very broad range of tolerance to multiple and fluctuating stresses can establish themselves under these conditions (Zakharova et al. 2013).

A decisive role in the growth and development of cyanobacteria and algae is played by appropriate light conditions, temperature and humidity, which are greatly associated with the distance from larger aquatic ecosystems and vegetation (Barberousse et al., 2006). In particular humidity probably represents the most important factor for the colonization of aeroterrestrial microalgae; moreover, when dried they can quickly recover if water becomes available again, e.g., after rain events. This ability explains well the ecological success of phototrophs in thriving on building facades and roof tiles in urban areas (Häubner et al., 2006).

The access to mineral compounds and adequate substrate pH are also important (Grbić et al., 2010) and the stone substratum itself may act as a putative source of minerals together with the air chemistry that may provide inorganic and organic compounds (Villa et al. 2015).

Atmospheric gases, aerosols, pollutants and particulates can be accumulated in biofilms and serve as nutrient sources as well as inoculum (Warsheid and Braams, 2000).

Although the number of eukaryotic studies is limited, algal and fungal communities on stone revealed a lower diversity in biofilms on stone surfaces compared with those in most natural systems (Gorbushina and Broughton 2009; Cutler et al 2013). Which particular microbial community dominates may depend on the substrate, the atmosphere, and abiotic stresses (Ranalli, et al., 2009). Organic components in the rock substrate or atmosphere also encourage chemoorganotrophic development, which in turn leads to further organic enrichment of the system through biomass production, exudation and exopolymer synthesis (Warsheid and Braams, 2000).

1.1.2 Establishment of a subaerial biofilm

Colonization is one of the first steps leading to the subsequent formation of a biofilm on a material, resulting at best in a reduction of its performance and, at worst, in its destruction. Hamilton and Characklis (1989) described the phases of biofilm development as follows: (l) the transport of organic molecules and cells to the surface, (2) the adsorption of organic molecules to give a 'conditioned' surface, (3) the adsorption of cells to the conditioned surface, (4) the growth of adsorbed cells with associated synthesis of expolymeric substances (EPS).

Biofilms are composed primarily of microbial cells and EPS, that may account for 50% to 90% of the total organic carbon of biofilms (Flemming et al., 2000) and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The EPS matrix is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. Its production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote EPS synthesis (Sutherland 2001) It is known that bacteria embedded in the biofilm matrix are remarkably more tolerant to biocides, up to 1000-fold relative to planktonic cultures of the same bacterial strains, depending on the species–drug combination (Davies 2003).

After the establishment of a biofilm community, a highly degraded stone surface with subsequent alteration of the physical condition of the rock, provide appropriate conditions for the germination of reproductive structures from higher organisms. The formation of a ‘‘proto-soil’’ enables the growth of cryptogams (mosses and ferns) and higher plants (Lisci et al., 2003).

1.2 Biological weathering of stone substrata

Although ineluctable, stone weathering depends on its mineral composition and environmental conditions, mostly influenced by climate and human activities (Warscheid and Braams 2000). A large part of the world's most precious cultural heritage and artworks are made of stone with a finite life, and they are slowly but irreversibly disappearing (Schreerer et al. 2009).

For the first time in 1965, Hueck (1965, 1968) defined biodeterioration as "*any undesiderable change in the properties of a material caused by the vital activities of organisms*"; till then, the weathering of stone monuments and artworks was attributed to physical agents, while later it became more and more clear that biofilms play an active role in stone decay.

1.2.1 Biodeterioration

Biofilms are particularly evident in altering the appearance of stone structures (Cutler and Viles, 2010; Gaylarde and Gaylarde, 2005) with fungi considered to be the most important chemoorganotrophs (De la Torre et al.,1993, Koestler et al., 1997).

Microbial growth on stone surfaces follows the complex topography of the substrate and generates a patchy biofilm that spreads between the mineral grains filling depressions, fissures, and inter-granular spaces (Gorbushina 2007). There are, however, trends in colonization, which are related to the physical properties of materials such as roughness and porosity (Barberousse et al., 2007). The solid surface may have several characteristics that are important in the attachment process. Characklis et al. (1990) noted that the extent of microbial colonization appears to increase as the surface roughness increases. Tolker-Nielsen and Molin (2000) noted that every microbial biofilm community is unique although some structural attributes can generally be considered universal.

Stone inhabiting microorganisms may grow on the surface (epilithic), in crevices and fissures (chasmolithic), or may penetrate some millimetres or even centimetres into the rock pore system (endolithic)(Tiano, 2002b), thereby gaining protection from environmental extremes (Hoppert et al., 2004). The pore spaces in rocks, that is, the endolithic environment, can also host photosynthesis-based communities that are often thought to be among the simplest ecosystems known (Walker and Pace, 2007).

Typical mechanisms of microbial weathering involve physical and biochemical destruction. Generally speaking, biodeterioration process can occur due to:

(1) Mechanical processes, where the material is damaged as a direct result of the activity of an organism, such as its movement or growth. An example of this form of biodeterioration is the damage caused to cabling as a result of insect or rodent attack.

(2) Chemical assimilatory biodeterioration, perhaps the most common form of biodeterioration. It occurs when a material is degraded for its nutritive value. The breakdown of cellulosic materials by cellulolytic micro-organisms, is an example of this type of biodeterioration.

(3) Chemical dissimilatory biodeterioration, which occurs when meta- bolic products damage a material by causing corrosion, pigmentation, or by the release of toxic metabolites into a substance. The poisoning of grain by mycotoxins is an example of this process.

(4) Soiling/biofouling, the form of biodeterioration which occurs when the mere presence of an organism or its excrement renders the product unacceptable. The biofouling of ships' hulls, the formation of slime in fuel lines and corrosion within water pipelines are examples of this form of biodeterioration.

Physical mechanisms of bioweathering include penetration by filamentous microorganisms (for example, certain actinobacteria, cyanobacteria, algae, fungi) along points of weakness, or direct tunnelling or boring, especially in weakened or porous substrata (Hoppertet al., 2004; Jongmans et al., 1997; Lian et al., 2008).

Many cyanobacteria, not necessarily filamentous, have also been shown to have a boring ability (Cockell and Herrera, 2008). Organisms that actively bore (euendoliths) widely occur in cyanobacteria, red and green algae and fungi (Cockell and Herrera, 2008). Other physical effects on substrate integrity can be due to cell turgor pressure, and exopolysaccharide and/or secondary mineral formation (Barker and Banfield, 1996).

The production of efflorescences (‘salting’) involves secondary minerals that are produced through the reaction of anions from excreted acids with cations from the stone. Such secondary mineral formation can cause blistering, scaling, granular disintegration and flaking or ‘spalling’ of outer layers. This may often be a major mechanism of stone decay (Wright, 2002).

Phototrophs inhabiting anthropogenic substrates thereby contribute to their rapid biodeterioration (Tomaselli et al., 2000; Crispim and Gaylarde, 2004; Samad and Adhikary, 2008). They produce photosynthetic pigments, which change the color of the substrates on which the cyanobacteria and algae grow. This adversely affects the aesthetic value of buildings and cultural monuments (Grbić et al., 2010; Stupar et al., 2012). When humidity changes, the hydration and volume of algal cells are also modified, causing structural microdamages to substrates (Hauer, 2010). Many phototrophs are capable of dissolving compounds contained in a substrate and penetrating into it, causing mechanical erosion (Brehm et al., 2005; Crispim and Gaylarde, 2004). During the metabolic activity of the algal cells, various types of inorganic and organic acids are produced and algae secrete them into the external environment, causing chemical deterioration of substrates (Gaylarde and Morton, 1999; Stupar et al., 2012).

For instance, aerobic microorganisms produce respiratory carbon dioxide, which becomes carbonic acid and contributes to dissolution of stone and soluble salt formation (Griffin et al., 1991; Wakefield & Jones, 1998). The precipitation of calcium salts on cyanobacterial cells growing on limestone suggests the migration of calcium from neighbouring sites (Arino et al., 1997; Crispim & Gaylarde, 2005). In addition, the production of organic acids such as lactic, oxalic, succinic, acetic, glycolic and pyruvic has been found and associated with the dissolution of calcite in calcareous stones (Danin & Caneva, 1990; Caneva et al., 1992). Endolithic photosynthetic microrganisms actively dissolve carbonates to enable penetration into the stone, enhancing stone porosity (Fernandes, 2006). Furthermore, the slimy surfaces of microbial biofilms favor the adherence of airborne particles (dust, pollen, spores, carbonaceous particles from combustion of oil and coal), giving rise to hard crusts and patinas (Saiz-Jimenez, 1999).

1.2.2 Bioreceptivity

Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. In order to explain the particular and specific interactions that occur among microrganisms and different substrata, Guillitte (1995) introduced the definition of bioreceptivity, explained as "*the aptitude of a material (or any other inanimate object) to be colonized by one or several groups of living organisms without necessarily undergoing any biodeterioration*". The word

‘colonize’ is important since it indicates that conditions for harbouring, development and multiplication have to be met and excludes the ability of a material to receive living organisms in a transient and fortuitous manner. It implies that there is an ecological relationship between the material and the colonising organisms.

The susceptibility of stone and mineral-based material to bioweathering is influenced by chemical and mineralogical composition, physical form, and geological origin (Hutchens, 2009; Turick and Berry 2016).

The presence of weatherable minerals in stone such as feldspars and clays may provide points of weakness and significantly increase susceptibility to attack (Warsheid and Braams, 2000).

CHAPTER 2
STATE OF THE ART

2.1 Traditional and modern methods for the characterization of a subaerial biofilm

One of the most remarkable aspects in the study of biofilms is the identification of the phototrophic and heterotrophic components and the analysis of the hidden biodiversity of these consortia, together with the reciprocal organization and the architecture of the community. At this aim, for a long time the main focus of researchers has been dedicated to the isolation and cultivation of microrganisms and their identification through their morphological features.

Moreover, culture media used to isolate environmental microbes include saccharides, proteins and vitamins in concentrations, which do not occur under environmental conditions, enabling the development of fast-growing species. Nevertheless, microscopy is still considered an useful instrument to investigate biofilm composition, especially CLS-M. Confocal laser scanning microscopy in fact has been largely used for the understanding of the relationships among microrganisms in a biofilm or between microrganisms and substratum (Sommerfeld Ross et al., 2014).

Although the protocols for electron microscopy can generate artifacts and even destroy the samples, the variant ESEM is low-vacuum scanning electron microscopy technique for biofilm that enables imaging of hydrated specimens (Little et al., 1991). Zammit et al., (2011) used Energy Dispersive X-Ray Spectroscopy (EDS) coupled to Environmental Scanning Electron Microscopy (ESEM) to study mineral structures formed by bacterial and microalgal biofilms growing on the archaeological surface in Maltese Catacombs.

In the last three decades, the advance of molecular biology techniques made possible to study new aspects of biofilm ecology and community structures, especially thanks to the enlargement of genomic databases united with the broad use of barcoding markers. Most common molecular markers used for the identification are the genes

encoding for the 16S rRNA for prokaryotes and 18S rRNA for eukaryotes (Gonzalez and Saiz-Jimenez 2005; Dakal and Arora, 2012). They are present in all prokaryotic and eukaryotic organisms and structurally and functionally conserved; most importantly, they alternate highly conserved and variable regions, which allow the fingerprinting (Rastogi and Sani, 2011). Also internal transcribed spacer (ITS) region, located between 18S and 28S rRNA, can be used to determine algae and molds. DNA extraction coupled with PCR reaction regarding barcoding genes allowed to identify microrganisms efficiently; Macedo and colleagues (2009) for instance report several years of identification of phototrophic species returning a picture of biodiversity of phototrophic species dwelling on monuments in the area of Mediterranean basin. Moreover, a number of techniques exist which were implemented on PCR for community studies.

2.1.1 Molecular techniques for the study of microbial communities

In denaturing gradient gel electrophoresis (DGGE), the PCR-amplified molecular markers are separated in polyacrylamide gels containing a linearly increasing gradient of denaturants such as urea and formamide. In these methods, DNA fragments of equal length, but with different base-pair sequences can be separated (Muyzer et al., 1993; Muyzer and Smalla, 1998). Temperature gradient gel electrophoresis (TGGE) relies on the same principle as DGGE, except that temperature gradient is used instead of a mixture of urea and formamide (Rastogi and Sani, 2011). Migration of DNA fragments in DGGE and TGGE is based on the electrophoretic mobility of a partially melted DNA molecule in the polyacrylamide gel. The DGGE/TGGE techniques have been applied to evaluate the structure of microbial communities consisting of bacteria (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2001), cyanobacteria (Cappitelli et al., 2009; Gaylarde et al., 2012), archaea (Rölleke et al., 1996) and fungi (Carmona et al., 2006; Giacomucci et al., 2011) sampled from walls, cave paintings, stained window glasses and several others substrata.

ARDRA is a useful method for rapid monitoring of microbial communities over time, or comparing biodiversity in response to changing environmental conditions. In this assay ribosomal RNA genes are amplified by a PCR reaction from environmental DNA. Later the products are digested into specific DNA fragments with tetracutter restriction endonucleases and separated on agarose or polyacrylamide gel. One of the

major limitations of ARDRA is that it provides little or no information about the type of microorganisms present in the sample (Gich *et al.*, 2000). The ARDRA method was used to evaluate the biodiversity of cyanobacteria on stone monuments in the Boboli Gardens in Florence (Tomaselli *et al.*, 2000).

T-RFLP protocol involves the amplification of the 16S rRNA gene by using the PCR method. The major difference from ARDRA is that one or both primers used during the PCR reaction are 5'-fluorescently labeled (Liu et al., 1997). Amplified target genes are digested with restriction enzymes and the obtained terminal restriction fragments (TRFs) are separated by using capillary or polyacrylamide gel electrophoresis in an automated DNA sequencer. Only the terminally fluorescently labelled restriction fragments are detected, the analysis of the structure of microbial communities (Rastogi and Sani 2011, Cetecioglu et al., 2012). Cutler et al (2015) used T-RFLP for the understanding spatial heterogeneity in the biodeterioration of stone as the observed patchiness of fungal and algal varieties is likely to be correlated with centimetre-scale variation in stone degradation and soiling.

Single strand conformation polymorphism (SSCP) is based on separation of the same-length DNA fragments according to their differences in mobility in polyacrylamide gel caused by differences in the secondary structure of folded DNA. PCR products are denatured and this is followed by electrophoretic separation in a non-denaturing polyacrylamide gel (Cetecioglu et al., 2012).

Automated ribosomal intergenic spacer analysis (ARISA) is a high resolution, culture-independent method suitable for an analysis of biodiversity and richness of microbial communities. Developed by Fisher and Triplett 1999, the PCR fingerprinting technique is based on the size and nucleotide sequence variability of the intergenic spacer region (IGS) present between the small (16S) and large (23S) ribosomal subunits (Cardinale et al., 2004). The IGS fragments are analysed by an automated capillary electrophoresis system containing a laser detector. The ARISA technique was applied by Cuzman and coworkers (2010) to study the species structure of biofilms formed in historic fountains in Italy and Spain.

MDA consists of a pre-PCR amplification strategy using random hexamers and fi29 DNA polymerase. The reaction is carried out at a constant temperature of 30°C and

generates non-specific genomic amplification products. In a second step the newly generated DNA serves as template for a PCR reaction using 16S-rRNA-specific primers, a thermostable DNA polymerase, and standard thermal conditions. This procedure results in the amplification of 16S rRNA gene fragments from natural samples at concentrations 10-fold lower than used in standard PCR amplifications (Gonzalez et al., 2005).

2.1.2 Methods for microbial biomass estimation

A biomass estimation of biofilms can be used to define the extent of microbial growth on a substrate. Several methods can be applied to this aim: HPLC chromatography is broadly used for the determination of the biomass, both for the heterotrophic and the autotrophic components in a biofilm through the quantification of ergosterol and / or chlorophyll a, even from solid substrata as showed by Gors and coworkers (2007).

The estimation of algal biomass has also been successfully performed with LIBS (laser induced breakdown spectroscopy). This is a spectrochemical analytical technique LIBS, which is based on generating a laser-induced plasma (LIP) by high energy laser pulses and subsequent time-resolved spectral analysis of the LIP emission, can be used to analyze materials in any state of matter (Cremers and Radziemski, 2006; Miziolek, et al., 2006.). An interesting application is the analysis of algal biomass for industrial biotechnology (Pořízka, 2012; Martin et al., 2017).

Flow cytometry has been successfully applied for years to analyze phytoplanktonic communities. Recently it has been also used for the quantification of phototrophic and heterotrophic components of solid biofilms from Moidons caves (France) and also for bacterial biofilms to test anti-fouling coatings in marine environment (Borderie et al., 2016; Camps et al., 2014). This powerful analysis permitted to discriminate microrganisms on the basis of DNA content and/or quality of the pigments.

2.2 In vitro experiments for the understanding of biofilm ecology

In general terms, deterioration can be defined as a loss of structural capacity with time as a result of the action of external agents or material weakening (Sanchez-Silva and Rosowsky, 2008).

The study of biodeterioration and weathering effects on material decay requires a combination of microbiological, surface analysis and materials characterization techniques. Biodeterioration evaluation typically involves the identification of the major types of microorganisms present in biofilms formed on building materials united with the microscopical observation of the interface biofilm/ material. Moreover, the elemental and mineral analysis of the damaged material and the correlation between the morphological and metabolic properties of the identified organisms, the morphology of the decay and the chemistry of the altered material are relevant for the assessment of biodeterioration (Herrera and Videla, 2009).

A better understanding of the mechanisms involeved in bio-weathering and its effects on materials properties is still needed. At the moment, tests to study biodeterioration of building materials exist, including a broad range of materials and microrgansims.

Among them some were developed without accelerated weathering of the matrix leading to longterm experiments (Ohsima et al., 1999; Urzı` and De Leo, 2007), while some other aim on qualifying aesthetic damage of external wall surface exposed to biofilm colonisation (Escadeillas et al., 2007).

Natural stone exhibits a wide range of mineral composition, texture and structure. Therefore, the physical and chemical properties of different types of stone are extremely variable, resulting in stone with widely different abilities to resist weathering (durability). Decay of stone materials as a result of their interaction with the environment can lead to loss of the essential messages of the architectural object, in terms of cultural or artistic values. The most immediate consequence of this interaction is chemical and physical alteration followed, in most cases, by biological colonization.

The degree of biological colonization of a stone surface depends not only on environmental factors but also on the intrinsic properties of the material (Guillitte, 1995), thus two different types of stone may undergo different degrees of colonization under the same environmental conditions.

Several studies have investigated the bioreceptivity of stone materials (among the others e.g. Saiz-Jimenez et al., 1995; Urzì and Realini, 1998; Prieto and Silva, 2005; Prieto et al., 2006; Cámara et al., 2008, 2011; Favero-Longo et al., 2009; Giannantonio et al., 2009).

Such experimental simulations, commonly used in ecological studies, are of great interest for the particular case of cultural heritage materials, since they allow experimental manipulation of the microbial ecosystem without the need for sampling and subsequent damage to cultural assets.

CHAPTER 3
AIMS OF THE WORK

Monuments and façade of historical buildings can be optimal environments to study ecological relationships of stone communities together with the complex mineralogical properties of the substrata and the microclimatic parameters, that are demonstrated to play a key role in the establishment of subaerial biofilms (Benavente et al., 2008).
Cultural heritage in all its forms and expressions can host species of notable scientific interest and singular behavior. Only within the last three decades biodeterioration received serious attention from conservators and conservation scientists (Price, 1996; Schnabel, 1991). A thorough understanding of the factors and mechanisms involved in microbial biodeterioration is essential to develop appropriate methods for its control. Two main groups of microorganisms, algae and fungi, are known to colonize the external surfaces of buildings and monuments giving the surface a dirty, neglected and unsightly appearance (Perrichet, 1987). Both of them were studied in the present work, under several aspects.

Three main objectives were crosswisely pursued in the presented works: 1) the advance in isolation and identification methods for the identification of microrganisms; 2) the assessment of *in vitro* systems to study the early steps of microbial colonization and bioreceptivity of stone; 3) the assessment of new tools for the evaluation of subaerial biofilms.

In recent times molecular biology techniques have been necessary in order to understand composition and structure of subaerial biofilms, avoiding the cultivation and the isolation of single components. Neverthless the identification of microrganisms is not informative itself about the true composition of the mat, and no one of the present techniques used to this aim permits to obtain both qualitative and quantitative information. More than a deep description of all the microrganisms involved in a mat, often present just in little traces, an evaluation of the microbial community on the basis of its main actors may be needed. It's the author's thought that this kind of approach could be very helpful in the

study of biofilms for the extimation of bacterial, fungal and algal biomass both for direct in situ analysis and in vitro simulation.
Real time PCR (qPCR) is an extremely sensitive assay which allows the quantification of a target gene or a transcript; if coupled with internal fluorescent probes it can also be informative about differential targets within the template, ie. two splicing variants of a transcript. Moreover, fluorescent internal probes may be used in a multiplex reaction, determining relative levels of template for each target.
In this work it is presented a novel approach for the study of subaerial biofilms through the construction of three fluorescent internal probes, respectively designed on 16S, ITS and tufA genes. The work is presented in Chapter 4, as a paper in submission entitled "*Survey of relevant taxonomic groups for the design of qPCR primers and internal fluorescent probes for whole biofilm characterization*".

Besides, in Chapter 5 it is discussed the use of flow cytometry and species-specific primer design for the isolation and identification of Cyanidiales (Rhodophyta).
One of the issue in the sampling of microbial mats from lithic substrata is the subsequent isolation of microrganisms. In a survey at the ancient Stufe of Solfatara (Pozzuoli, Italy) were retrieved microrgansisms belonging to the Order Cyanidiales (Rhodophyta), which thrive in thermoacidic environments. Despite the ordinary methods applied to the isolation and identification of microalgae, Cyanidiales cannot be selected on differential media and their poor morphology hardly permits to distinguish among them. Cyanidiales are currently divided into three Genera, namely Cyanidium, Galdieria and Cyanidioschyzon.
In collaboration with the PRECYM platform at MIO Institute (University of Aix-Marseille) a cell-sorting strategy was developed in order to isolate and assess the purity of three collected samples. The results obtained by the flow cytometric analysis of the samples were then confirmed in a PCR assay with species-specific primers designed for this aim. The study of Cyanidiales, which have a peculiar tolerance to harsh conditions and are also capable of endolithic growth, may lead to new insights relevant to the colonization of stone substrata and their fissures and cavities, even when the environmental parameters would be prohibitive for the broad majority of microrganisms. The work is a paper in submission entitled "*A flow cytometry coupled to species-specific primers approach for the isolation and identification of Cyanidiales (Rhodophyta) sampled at Stufe of Solfatara (Pozzuoli, Italy).*"

Moreover, a series of in vitro experiments were performed both on Fungi (*Fusarium solani, Alternaria alternata*) and algae (*Oculatella subterranea*) to obtain a model that allows to evaluate both the aspects related to the colonizing attitude of microrganisms and the bioreceptivity of building materials toward some of the most common rock inhabitants.

These kind of experiments allow to produce a controlled environment in which light, nutrients, temperature, humidity, substratum and model organism may be alternatively changed to observe different response in colonization. It has been decided to focus on some features of subaerial biofilms that are poorly discussed in literature, such as the cryptoendolithic growth determination and the orientering of branchings during the growth, included the density of the architecture of the colony. To do so, several techniques were used, especially: light and metallurgical microscopy, fluorescence and confocal microscopy, counts of conidia, determination of ergosterol with HPLC, image analysis and statistics with opportune softwares.

Both lithotypes and microrganisms used for the experiments were chosen on the basis of sampling campaigns in some of the most significant archaeological sites in Campania (Italy), the archaeological sites of Oplontis and Pompeii. All the efforts on this aim were collected into three works in submission presented in Chapters 6, 7 and 8.

CHAPTER 4

Survey of relevant taxonomic groups for the design of qPCR primers and internal fluorescent probes for whole biofilm characterization

Survey of relevant taxonomic groups for the design of qPCR primers and internal fluorescent probes for whole biofilm characterization

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Abstract A survey of biodetergiogen microrganisms occuring on stone monuments in Europe has been performed. The obtained lists for eukaryotic algae, phototrophic and non-phototrophic bacteria and soil fungi were sorted by Genera and corresponding sequences in triplicate were downloaded by nucleotide database Genbank for a number of selected barcoding markers. On the basis of collected diversity, multiple nucleotide alignements were produced and primers were designed for a qPCR assay. The aim of the present study was to obtain accurate oligos for the characterization of subaerial biofilms. Primers were designed on conserved regions flanking a a variable region, specific for each of the studied groups of microrganisms. Standard curve for absolute quantification relative to each group were determined. Then, variable regions in the alignments were used to design fluorescent internal probes for qPCR aimed for a multiplex reaction in which relative abundance could determined.

Introduction

The identification of the phototrophic and heterotrophic components of subaerial biofilms is to date one of the most pursued aims of biofilm research. The advance of molecular biology techniques made possible to discover new aspects of biofilm ecology and community structures, primarily due to the enlargement of genomic databases together with the broad use of barcoding markers. Most common molecular markers used for the identification are the genes encoding for the 16S rRNA for prokaryotes and 18S rRNA for eukaryotes (Gonzalez and Saiz-jimenez 2005; Dakal and Arora, 2012). They are present in all prokaryotic and eukaryotic organisms and structurally and functionally conserved; most importantly, they alternate highly conserved and variable regions, which allow the fingerprinting (Rastogi and Sani, 2011). Also internal transcribed spacer (ITS) region, located between 18S and 28S rRNA, can be used to determine molds (Op De Beeck et al., 2014). DNA extraction coupled with PCR reaction regarding barcoding genes allowed to identify microrganisms efficiently. Beside the use of 18S marker for green algae identification, a number of plastidial markers have been proposed and established, above all rbcL and TufA (Hall et al., 2010; Saunders and Kucera, 2010; Du

et al., 2014).

Similarly for Cyanobacteria it has been proposed the use of cpcA (C-phycocyanin alpha chain) (Neilan et al., 1995; Miller and McMahon, 2011) and dinitrogenase reductase nifH genes for barcoding (Zehr and McReynolds, 1989; Poly et al., 2000).

In addiction, a number of techniques exist which were implemented on PCR for community studies, as ARDRA, DGGE and ARISA (Rastogi and Sani, 2011; Agrawal et al., 2015). However, they cannot be reliable for quantitative results and/or may be present problems in pattern visualization on agarose gel and other major limitations (Neilson et al., 2013; Rastogi and Sani, 2011; Agrawal et al., 2015). For this reason, new tools are required in order to describe composition and relationships of microbial mats.

The aim of the present study is to describe microrganism involved in biofilm formation in Europe and Mediterranean countries; on this basis, the authors also propose the use of novel designed oligos for whole characterization of subaerial biofilms, with possible application in a multiplex qPCR assay with fluorescent internal probes.

Material and methods

Survey of identified biodeteriogens

Case studies and reviews regarding biodeterioration of stone monuments in Europe and Mediterranean countries were collected, and all identified microrganisms were listed. Compilation proceeded by genera, that were annoted with the available nucleotide sequences for selected markers (Supplementary materials Table 1-2-3-4). Diatoms and red algae are scarcely represented in these kinds of biofilm and were excluded by marker selection (Supplementary materials Table 5).

Multiple nucleotide alignment for the selected markers

Seven candidate molecular markers (Bacteria: cdcA, 16S, NifH; Green microalgae: rbcL, tufa, 18S; Fungi: ITS1) were chosen for the three selected groups of microrganisms. For each candidate marker at least three sequences were downloaded by Genbank nucleotide database for each Genus, plus additional sequences of related genera not retrieved on monuments, in order to confirm the conserved regions and find selective variability in non-conserved ones. Seven nucleotide multiple alignments were generated with UGENE software v.1.27 (Okonechnikov et al., 2012). The alignments were then trimmed and

adjusted by eye, and the primers were designed in regions showing selective differences according to species attribution and position similarity score into the alignment. Original alignments and side information are available in Supplementary materials. Primers were designed in order to obtain amplicons of maximum size of 200bp. Primers have been located in regions of 100% nucleotide conservation which contained a sequence variable selectively for the organisms of interest, suitable for designing an internal fluorescent probe. *In silico* PCR simulation were performed with Amplify4 software v.0.9.5 (Engels, 2015) that also provided the annealing temperature for each primer. The oligos were synthesized by IDT Company.

DNA extraction and PCR

The DNAs were extracted by ACUF collection (www.acuf.net) strains (Supplementary Materials Table 6) with CTAB DNA extraction (Doyle and Doyle, 1987). PCR were carried out in a 25µl aliquots containing approximately 50ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x buffer), supplemented to give a final concentration of 2.5mM MgCl2, 1.25U of Taq polymerase (EconoTaq, Lucigen), and 0.5pmol of each primer. Amplifications were run in a Applied Biosystem 2720 thermal cycler. The profile used was 10 min at 95°C, 15 cycles of 95°C for 30s, 48°C for 30s, and 72°C 30s with annealing increasing of +0,5° at each cyle, followed by 20 cycles of 95°C for 30s, 56°C for 30s, and 72°C 30s and a final elongation step of 10mins at 72°C. Finally, 1.5% (w/v) agarose gel electrophoresis was used to examine the reaction products. All four couples of primers were used in four different PCR reactions targeting four different mixtures of DNAs in order to test group-specificity.

Standard qPCR curve for absloute DNA quantification

Six dilution series of mixed DNAs at eight different concentrations has been used to establish a standard curve for determining the initial starting amount of the target template in experimental samples and for assessing the reaction efficiency for each selected primer couple. This procedure has been followed for each group of microrganisms for the four selected markers, 16S, rbcl, tufa, ITS. The PCR reactions were carried out using the RealAmp™ SYBR qPCR Master (GeneAll® Biotechnology), 1,5 pmol of each primer and 1µl of DNA-dilution. Amplification reactions were performed in a total reaction volume of 10 µl in a 96-well PCR-Plate (StarLab, Hamburg,

Germany) on the Applied Biosystems 7500 (Foster City, CA, USA) with the following program: 10 min at 95°C (denaturation and Taq polymerase activation), an amplification program of 45 cycles at 95°C for 15s, 60°C for 20s, and 72°C for 31s. The threshold cycle value (Ct), which refers to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the instrument software as the first maximum of the second derivative of the curve. Reaction efficiency was evaluated with LRE analyzer 0.9.10.

Fluorescent internal probes design

After that selected primers were tested in PCR and qPCR, fluorescent internal probes were designed. The choice for the opportune fluorophores and quenchers has been driven by the possibility to use the probes in a multiplex reaction, in compatibility with a StepOnePlus™ Real-Time PCR System instrument (Foster City, CA, USA). Four probes have been designed with the Oligo Architect™ online software (http://www.sigmaaldrich.com) for three barcoding markers, namely TufA, ITS and 16S. Two probes were designed for Fungi, in order to detect Ascomycota/Zygomycota and Basidiomycota phyla. LNA were inserted in order to increase the melting temperature of each probe, so to reach 10°C over the respective primer couple. The probes were synthesized by Sigma-Aldrich Company.

Results and discussion

Diversity of biodeteriogens and evaluation of barcoding markers

Organisms involved in subaerial biofilm formation represent a huge variety of microalgae, cyanobacteria, soil fungi and bacteria (Salvadori and Municchia, 2016; Isola et al., 2016). Our survey could not assess a defined majority that is primarily involved in biodeterioration. This evidence could mean that there is no precise involvement of one ore more species in biofilm formation, thus the participation of ubiquitous soil and freshwater microrganisms occurs in a way that could be primarily influenced by environmental parameters or metabolic features. Phototrophic bacteria all belong to the phylum Cyanophyceae, but their diversity in subaerial biofilms is mainly restricted to three Orders, i.e. Nostocales, Chrococcales, Oscillatoriales. Eukaryotic microalgae are mainly represented by the Chlorophyta phylum (93%); the extant 7% is due to the contribution of Charophyta phylum, by the Orders Zygnematales and Desmidiales.

Similarly, soil Fungi show a broad majority of organisms of the Ascomycota phylum (88%), followed by Basidiomycota and Zygomycota. However, even at the higher rank of Order, both Chlorophyta and Ascomycota retrieved in subaerial biofilms showed a wide diversity, with the presence of 12 different Orders of Chlorophyta and 17 different Orders of Ascomycota. Since the investigation of subaerial biofilms has been historically object of the study of phycologists and mycologists, Bacteria are somehow less investigated; moreover, non-phototrophic Bacteria may not be viable and cultivable. Data collected by literature reports were also analyzed for heterotrophic bacteria, and as expected variability is already notable at the level of phylum. Diatoms also scarcely retrieved in subaerial biofilms, although it is seldom reported the presence of Naviculales (*Navicula, Diadesmis*), Melosirales (*Melosira*) and Achnantales (*Achnanthes*). All the graphics are shown in Figure 1.

Barcoding markers are widely used for the identification of microrganisms; nonetheless, their use for the quantification of microrganisms is strictly limited to some particular cases (Pavón et al., 2011) and no suitable primers are available in literature for the specific aim of determining biofilm composition. Due to the availability of sequences in the databases, only four markers by the firstly selected seven were used for qPCR assays, i.e. Rbcl, TufA, 16S, ITS. Moreover, the chosen barcoding markers responded to the prerequisites in alternation of conserved and variable regions. Design proceeded in a way that is discriminating for the three major groups of microrganisms investigated. BLAST search and *in silico* PCR simulations were used to assess the specificity for the chosen templates, whereas classical PCR assays determined the real specificity on the selected DNAs, without cross amplification for each of the selected groups. Also, non-amplification for human and vertebrates was checked. The obtained oligos are reported in Table 1.

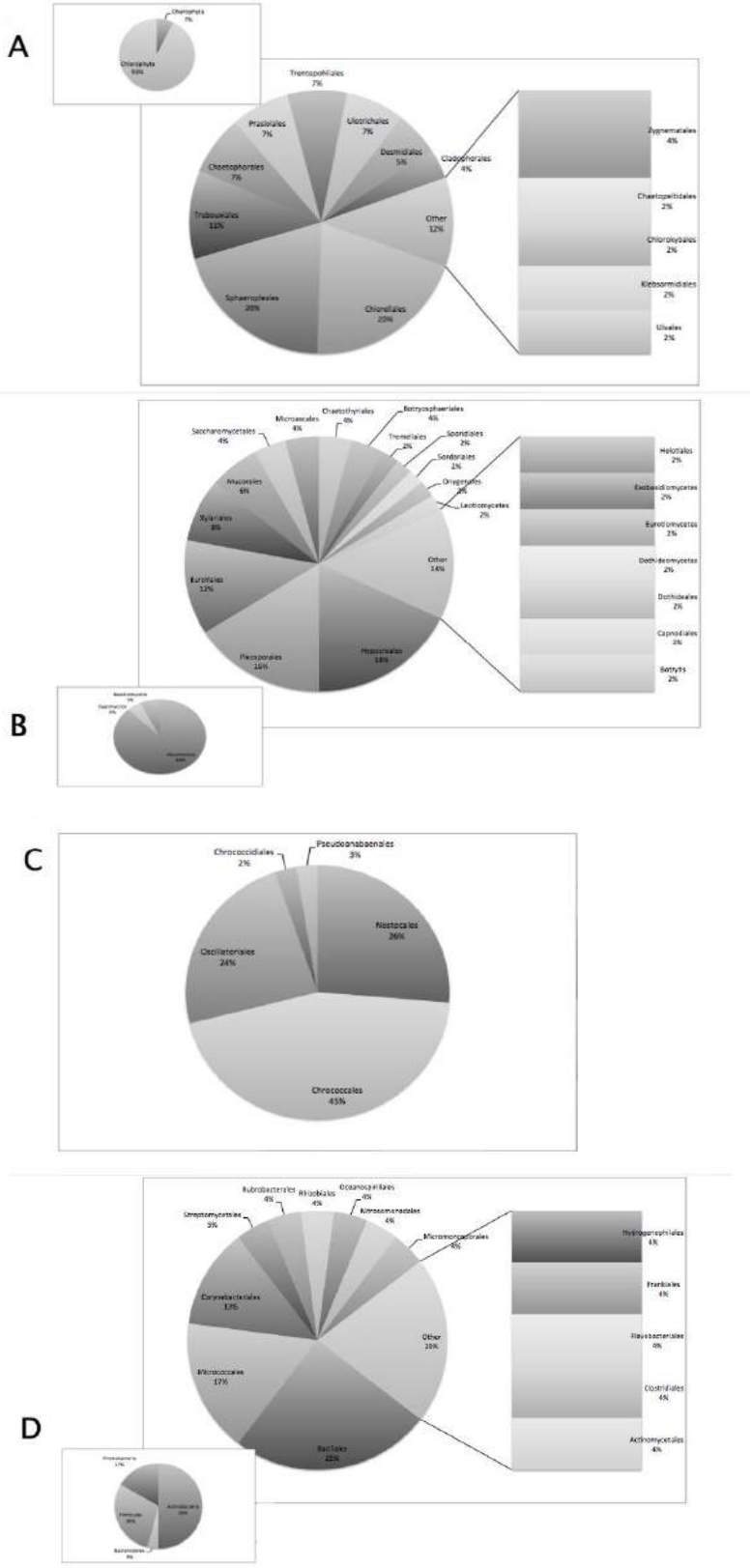

Fig. 1 - Phyla and Ordines reported diversity for A) eukaryotic microalgae B) soil fungi C) phototrophic bacteria D) non-phototrophic bacteria.

Table 1 - Selected barcoding markers and oligos for qPCR amplification of eukaryotic algae, bacteria and fungi from subaerial biofilms.

Marker	Oligo name	Sequence	Length
TufA	Tufa_F	5′)–)GCTGCTCAAATGGATGGTGC)–)3′	23bp
	Tufa_R	5′)–)TCATATTTATCTAAAGTTTCACG)–)3′)	20bp
RbcL	rbcl_F	5')8)TTYATGCGTTGGAGAGAYCG)8)3'	20bp
	rbcl_R	5')8)GTGCATAGCWCGGTGAATRTG)8)3'	21bp
ITS	ITS_F	5')8)CTTTCAACAACGGATCTCTTG)8)3'	21bp
	ITS_R	5')8)TTCAAAGATTCGATGATTCAC)8)3'	21bp
16S	16s_F	5')8)AGGATGCAAGCGTTATCCGG)8)3'	20bp
	16s_R	5')8)AATCCCATTCGCTCCCCTAG)8)3'	20bp

Standard curves and amplification efficiency

Absolute quantification describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve. Quantification is performed by comparing Ct values for unknown samples against this standard curve or, in the case of relative quantification, against each other, with the standard curve serving as an efficiency check. Ct values are inversely related to the amount of starting template: the higher the amount of starting template in a reaction, the lower the Ct value for that reaction. To determine the sensitivity of the real-time PCR system developed, standard curves relating Ct values and the logarithm of DNA were built (Fig. 2). The amplification efficiency was initially assessed by the slope of the standard curve, with the formula E=10^(-1/slope). The slopes of the linear equations were considered not reliable (>100%). It is reported that overestimation in reaction efficiencies may indicate pipetting errorsor contaminations (González-Salgado et al., 2009). However, it is the first time in which a standard curve is derived by mixed DNAs template; for this reason more than the exponential character of PCR reaction (i.e. primer efficiency) of the reaction, a Linear Regression of Efficiency was performed with LRE analyzer 0.9.10 (Rutledge and Stewart, 2008; Rutledge 2011). Standard curves were used to indirectly quantify mixed DNA specimens from in vitro experiments (*data not shown*). In Figure 2 the obtained standard curves are shown.

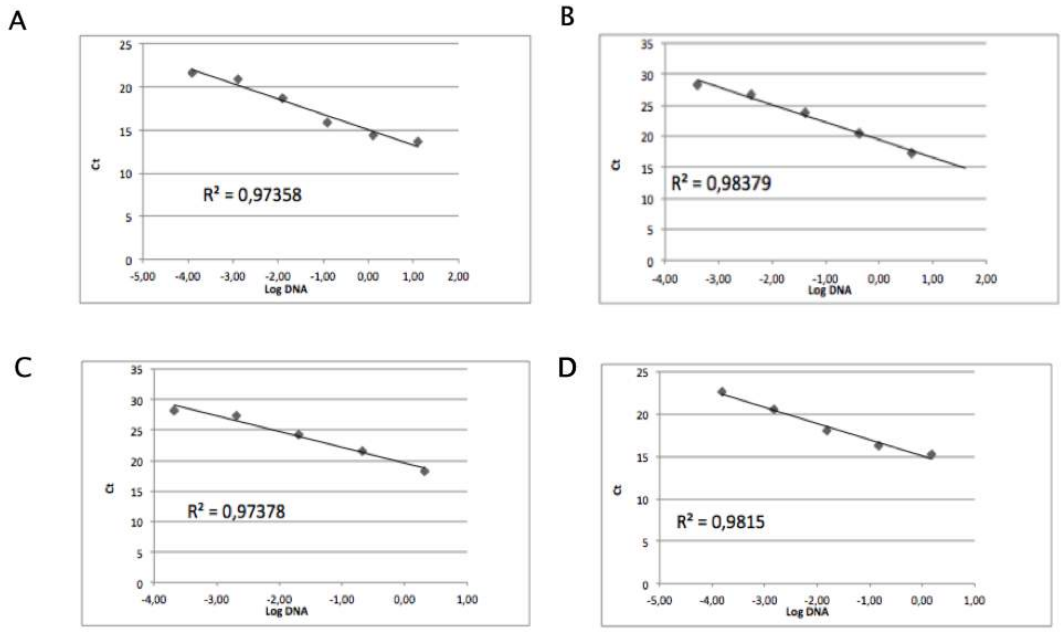

Fig. 2 - Standard qPCR curves from mixed DNAs coming from Fungi (ITS) (A), Bacteria (16S) (B) and eukaryotic algae with rbcL and tufa (C and D).

Table 2 - List of the novel-designed fluorescent internal probes. Fluorophores and quenchers were chosen to be compatible in a multiplex reaction. Letters in square brackets symbolize LNA nucleotides.

Marker	Oligo name	Sequence	Length
TufA	TufA probe	(JOE) 5' -YTTAAAYA[+A][+A][+G][+A]AGAYCAAGT - 3' (BHQ-1)	21bp
ITS1-ITS2	ITS-1Z probe	(FAM) 5' -TAG[+C]AAA[+G]T[+G][+C][+G]AT[+A]A[+C]TAG - 3' (BHQ-1)	20bp
ITS1-ITS2	ITS-2A probe	(FAM) 5' - CAGCG[+A][+A][+A][+T][+G][+C][+G][+A]TAAGTAA - 3' (BHQ-1)	20bp
16S	16S-eubat probe	(TAMRA) 5' - GTGTAGCG[+G]T[+G]AAATGCGTAG - 3' (BHQ-2)	21bp

Conclusion

In recent times molecular biology techniques have been necessary in order to understand composition and structure of subaerial biofilms, avoiding the cultivation and the isolation of single components. Neverthless the identification of microrganisms is not informative itself about the true composition of the mat, and no one of the present techniques used to this aim permits to obtain both qualitative and quantitative information.

More than a deep description of all the microrganisms involved in a mat, often present just in little traces, an evaluation of the microbial community on the basis of its main actors may be needed. In this work we present a novel approach for the study of subaerial biofilms through the construction of qPCR primers and fluorescent internal probes for the characterization and quantification of whole biofilms. Real time PCR (qPCR) is an extremely sensitive assay which allows the quantification of a template; if coupled with

internal fluorescent probes it can also be informative about differential targets within the template, ie. groups of phylogenetically distinct microrganisms. Moreover, fluorescent internal probes may be used in a multiplex reaction, determining relative levels of template for each target. The authors propose this kind of approach in the study of biofilms for the extimation of algae, molds and bacteria both for direct in situ analysis and in vitro simulation.

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CHAPTER 5

A flow cytometry coupled to species-specific primers approach for the isolation and identification of Cyanidiales (Rhodophyta) sampled at Stufe of Solfatara (Pozzuoli, Italy)

A flow cytometry coupled to species-specific primers approach for the isolation and identification of Cyanidiales (Rhodophyta) sampled at Stufe of Solfatara (Pozzuoli, Italy)

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Abstract The present paper proposes a polyphasic approach for isolation, identification and mantainance of collection strains of Cyanidiales. Species diversity of Cyanidiales (Rhodophyta) was investigated for biofilms retrieved on the Stufe of Solfatara (Pozzuoli, NA, Italy) using flow cytometry and four novel designed species-specific primers. Primers were built on rbcL gene for a PCR assay and tested on axenic Cyanidiales strains of ACUF collection. Three environmental samples were analyzed for their autotrophic components with flow cytometry, revealing the presence of *Galdieria sulphuraria* and *Cyanidium caldarium*. Relationships between flow cytometry results and PCR analysis were established for the isolation and identification of Cyanidiales. For the first time unicellular Rhodophyta were sorted with flow cytometry, allowing a sensitive and accurate separation among closely related Red Algae.

Introduction

Flow cytometry allows fast counting and optical analysis of individual particles, and it has also been adopted for species discrimination, since wide application of flow cytometry can be found for the analysis of phytoplanktonic communities (Ubelaar and Jonker, 2000; Trask et al., 1981). Only few attempts have been made to screen aeroterrestrial microalgae, and mostly aimed to follow up the development of biotechnological processes with axenic strains of microalgae expressing higher content of valuable biological compounds (Hyka et al., 2012). In the study of microrganisms inhabiting stone substrata and building materials also few studies are reported in which flow cytometry is applied, as for the identification of phototrophic communities in Moidons cave (Borderie et al., 2016). Nonetheless, to our knowledge in literature there is no reported attempt of separating with flow cytometry cells belonging to morphologically related aeroterrestrial microalgae.

Soils and rocks of thermo-acidic environments are frequently inhabited by large populations of unicellular microalgae belonging to Cyanidiophytina (Rhodophyta), in which can coexist members of the genera *Cyanidium*, *Galdieria* and *Cyanidischyzon*.

They were considered as a single species and described as *Cyanidium caldarium* and officially recognized as a member of the Rhodophyta only in 1958 (Hirose). Merola et al. (1981) erected the three genera Cyanidium, Galdieria and Cyanidioschyzon, differentiating *Galdieria* from *Cyanidium* on the basis of cell size, chloroplast structure, endospore number, and ability to grow in heterotrophic conditions . Nonetheless, the life cycles of *Cyanidum* and *Galdieria* present phases in which cell size and morphological features are overlapping. The fact that Cyanidiales thrive in the same habitats, sharing common traits of acidotolerance and thermophily (De Luca and Taddei, 1970) may explain the uncertainty about the taxonomy of the Cyanidiophyceae of pre-molecular era. With this premise, we have used an approach based on flow cytometry coupled with molecular analyses to point out cellular complexity and size in order to sort these two genera. Species-specific primer PCR is a popular technology for species identification due to its high accuracy, sensitivity and convenience (Aguirre et al., 2015). The plastid encoded rbcL gene has been widely used for phylogenetic inference in algae. Universal primers are not available for rbcL, although some primers can be used on a wide range of taxa (Lewis et al., 1997). Given that variable portions of the gene are small enough to be sequenced in a single reaction and a large amount of sequence data is available from a wide range of taxa through GeneBank it is possible to design lineage-specific primers (Hall et al., 2010). Currently, phylogenetic analyses based on rbcL sequencing support the division of the Cyanidiophytina into four distinct lineages that include seven species (Ciniglia et al., 2004; Yoon et al., 2004). The aim of this work is to find practical tests to assess the identity of natural samples collected in the volcanic site of Phlaegrean Fields, Naples, Italy. In these samples is frequent to find *Galdieria* species in association with *Cyanidium caldarium*, Four species-specific primers were designed in order to easily discriminate among the most frequent Cyanidiales species occurring in natural samples collected in Phlaegrean Fields. A cytofluorimetric and sorting analysis was conducted to discriminate among these species, and the identity of the sorted strains was confirmed by PCR with the above mentioned species-specific primers. This is the first reported attempt to sort Cyanidiales with flow cytometry, and to our knowledge also the first one among aeroterrestrial microalgae aimed to separate morphologically close species, followed by a proper molecular identification.

Materials and methods

Sampling site and used algal strains

The algae used in this work were sampled in the area of the Solfatara, (Pozzuoli, NA, Italy), which is an active volcanic area with low pH values to sulfuric acid gas emissions and temperatures above 50°C. The Stufe are two ancient caves dug into the side of the mountain on the north side of the volcanic area of Solfatara (Pozzuoli, Na, Italy) at the end of century XIX to build natural *sudatorii*. In a second time they were covered with masonry, on which the patina was sampled. Three samples were collected and named as S64, S20 and S16. Beside the sampled algae, eight strains, namely *Galdieria sulphuraria* ACUF004; ACUF005; ACUF006; *G. maxima* ACUF132 – ACUF425 – ACUF742; *Cyanidium caldarium* ACUF008 – ACUF019; *Cyanidioschyzon merolae* ACUF199 - ACUF732, belonging to ACUF collection (www.acuf.net) were used to develop sorting strategy and PCR assay with species-specific primers. All of them were firstly confirmed as Cyanidiales by their morphological characteristics (Pinto et al., 2003) and selected on solid agar Allen medium (Allen, 1959) acidified with 7‰ H2SO4. The strains were all maintained in a climate chamber at 37 °C and ca. 20–25 µmol photons m^{-2} s^{-1} under continuous light (Osram - Daylight).

Species-specific primer design

A total of 115 sequences belonging to different Cyanidiales (25 *Galdieria maxima*, 62 *G. sulphuraria*, 6 *Cyanidioschyzon merolae*, 16 *Cyanidium caldarium,* 6 *Cyanidium cave.* See Supplementary materials, Table A for the Accession ID) were downloaded by Genbank nucleotide database; rbcL gene multiple alignment was obtained with UGENE software v.1.27 (Okonechnikov et al., 2012). The alignment, consisting of 1179 sites, was then trimmed and adjusted by eye, and the primers were designed in regions showing selective differences according to species attribution and position similarity score into the alignment. *In silico* PCR simulation were performed with Amplify4 software v.0.9.5 (Engels, 2015) that also provided the annealing temperature for each primer. The oligos were synthesized by IDT Company.

Specificity and sensitivity test of the species-specific primers

The DNA was extracted with a modified CTAB DNA extraction protocol (Cutler et al., 2012). Then PCR was carried out in a 25 µl aliquot containing approximately 50ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x buffer), supplemented to give a final concentration of 2.5mM MgCl2, 1.25U of Taq polymerase (EconoTaq, Lucigen), and 0.5pmol of each primer. Amplification was run in a Applied Biosystem 2720 thermal cycler. The profile used was 5 min at 95°C, 33 cycles of 95°C for 30s, 48°C for 45s, and 72°C 40s, and a final elongation step of 10mins at 72°C.Finally, 1.4% (w/v) agarose gel electrophoresis was used to examine the reaction products.

The specificity of the four couples of species-specific primers was tested by performing PCR assays on ten strains of ACUF – algal collection (see above) previously isolated with serial dilutions and striking on agar plates. All four couples of primers were used in four different PCR reactions targeting four different DNAs of Cyanidiales. Sensitivity tests for the species-specific primers were carried out in a series of seven concentrations for each one of the four different species, ranging from 0.001 ng/mL to 50 ng/mL and a negative control. The same reaction mixture and cycling processes described above for DNA amplification were used for both the specificity and the sensitivity tests.

Flow cytometry and sorting of *Galdieria* and *Cyanidium* strains

FCM analyses and cell sorting were performed at the Regional Flow Cytometry Platform for Microbiology PRECYM (https://precym.mio.univ-amu.fr/) with a BD Influx MarinerTM (BD Biosciences, Franklin Lakes, NJ, USA) high-speed cell sorter equipped with three laser lines: 488 nm (200 mW, Sapphire, Coherent), 561 nm (75 mW, Jive, Cobolt), and 355 nm (100 mW, Xcyte, JDSU).Cells were characterized by five optical signals collected from the 488nm laser: 1) Forward scatter (FSC) related to cell size; 2) Side scatter (SSC) related to cell structure and shape; 3) Green fluorescence (FLG, $510 < \lambda_{488\ nm} < 550$ nm) related to SYBR Green nucleic acid staining; 3) Orange fluorescence (FLO, $565 < \lambda_{488\ nm} < 595$ nm) related to phycoerythrin; 4) Red fluorescence (FLR, $\lambda_{488\ nm} > 630$ nm) related to chlorophyll a. In addition, the chlorophyll a red fluorescence was also collected from the 355 and 561 nm ($FLR_{355\ nm}$, $\lambda > 630$ nm, $FLR_{561\ nm}$, and $\lambda > 630$ nm), allowing a better discrimination among the different

subgroups containing chlorophyll a. Cell sorting was performed on four gated regions, chosen on the basis of pigment content ratio and morphological features of populations with 1 drop pure setting mode.

Results

Specificity and sensitivity of species-specific primers

Four species-specific primer couples were successfully developed based on rbcL gene (Table 1). All the primers could specifically amplify the rbcL gene sequence of *G. maxima, G. sulphuraria, C. merolae* and *C. caldarium*. No cross-amplification was detected among Cyanidiales as well as non-specific amplification products. PCR with the species-specific primers in a series of seven concentrations for a single strain of each 4 species was conducted to test the sensitivity of the primers. The DNA template concentrations of the used strains were 0.001, 0.01, 0.1, 1, 10, 25, and 50 ng/mL. The results showed that all species-specific primers could amplify the positive electrophoresis band at 0,001 ng/mL. Additionally, all species-specific primers indicated that the electrophoresis bands in the agarose gel appeared clearer with increasing concentrations of the DNA template. Gel revelations for all specificity and sensitivity assays are available in Supplementary materials.

Table 1 – The species-specific primers for rbcL amplification of four Cyanidiales species

Species	Primer*	5'-3'	Length	Tm*(°C)	Target*fragment
Galdieria(maxima	GM_F	GCATGTGATGTCTATCGTGC	20	57	540bp
	GM_R	TTCAGCTCTCTTATAAATCTCC	22	52	
Galdieria(sulphuraria	GS_F	AGCAGCAGACTTATATAGAGC	21	51	541bp
	GS_R	ATTAGCACGTGCATACATTTC	21	55	
Cyanidium(caldarium	CyCALD_F	AGAGAAAGGTTCTTGTACGTG	21	54	433bp
	Cy_R	GCCCAGTCCATTTCAAAGAA	20	60	
Cyanidioschyzon(merolae	CM_F	ACGTCCATTATTAGGTTGTAC	21	51	444bp
	CM_R	GAAATGACACGGAAGTTG	18	52	

Flow cytometry and sorting of the sampled strains

Three samples from the Stufe were analyzed with flow cytometry. Two main populations were identified for the FSC and SSC parameters in two out of three samples. Although the profile of Chlorophyll a content and phycoeritrin was distinctively differentiated into two high-content and low-content populations, they were not univocally linked to the two populations identified on morphological parameters, but more likely related to the

cellular size of the cells.

In order to sort *Cyanidium* and *Galdieria*, four gates were designed, namely: CYA, POP1, POP2 and SPORES. Four ways sorting has been used in order to verify if an overlapping of the two genera occurs between the two populations identified for the FSC/SSC parameters and were based on variation in size and cellular complexity in addition to pigments content. This gating strategy has been used for the samples 020 and 064; sample 016 showed a single population, therefore it has not been sorted (Fig. 1).

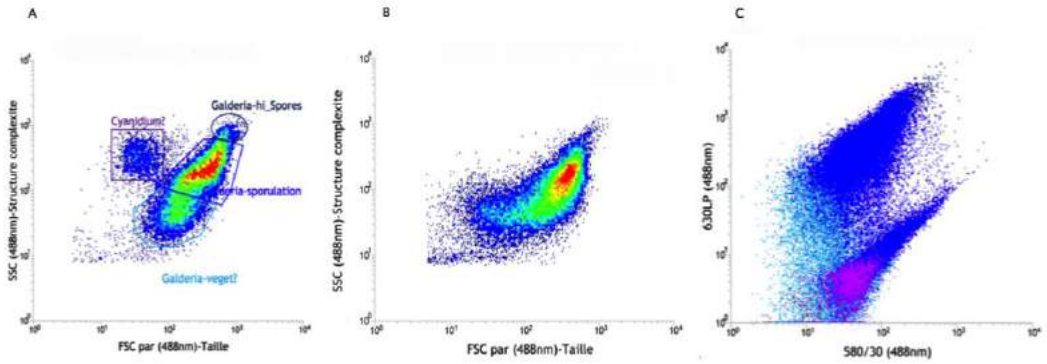

Fig. 1 – Flow cytometric analysis of environmental samples of Cyanidiales. **A.** FSC /SSC plot for the sample 064 and gating on hypothetical popultaions of Cyanidium and Galdieria. **B.** FSC /SSC plot for the sample 016, ungated; hypothetical Cyanidium population was not recorded. **C.** Phycoeritrin / Chlorophyll a fluorescence bivariate plot for the sample 064; in purple, hypothetical Cyanidium population as gated from FSC /SSC plot.

Molecular identification of the sorted algae

PCR assays with species-specific primer were performed in order to assess the identity of the three samples from the Stufe of Solfatara. Sample S016, which in cytofluorimetric analysis showed a single population for FSC/SSC parameters, was confirmed as a *G. sulphuraria*, and no other species were revealed when the DNA was used in combination with the other species-specific primers. On the other hand, PCR application to all four sorted population from the samples S020 and S064 confirmed that the sorted CYA population contained only *Cyanidium caldarium* whereas POP1, POP2 and SPORES sorted cultures were all belonging to the genus Galdieria, in particular *Galdieria sulphuraria* (Fig. 2).

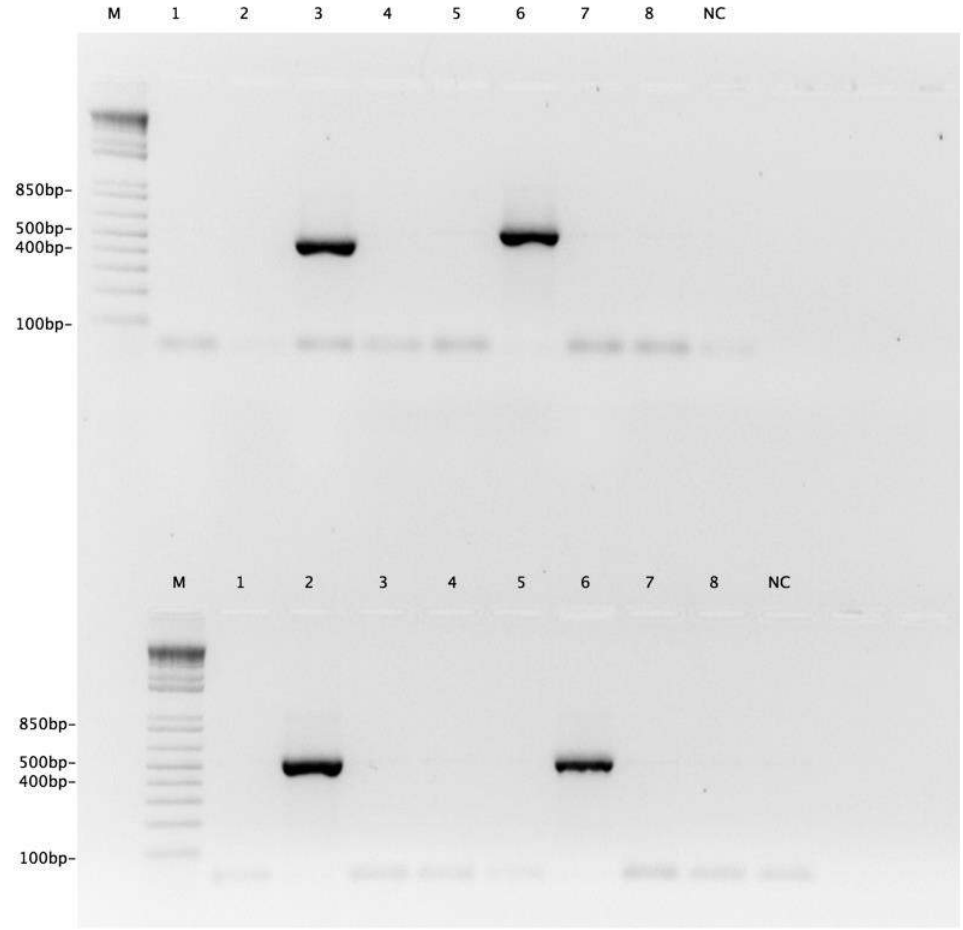

Fig. 2 - The application of species-specific primers for rapid species identification with electrophoresedrbcL PCR amplification products from four sorted cultures of Sample 064.**A.** M: DNA Marker 1kb plus; 1:*CYA (primer GM_F, GM_R)*; 2: *CYA (primer GS_F, GS_R)*; 3: *CYA (primer CYcald_F, CY_R)*; 4: *CYA (primer CM_F, CM_R)*; 5: *POP1 (primer GM_F, GM_R)*; 6: *POP1 (primer GS_F, GS_R)*; 7: *POP1 (primer CYcald_F, CY_R)*; 8: *POP1 (primer CM_F, CM_R)*; 9: Negative control).**B.**M: DNA Marker 1kb plus; 1:*POP2 (primer GM_F)*; 2: *POP2 (primer GS_F, GS_R)*; 3: *POP2 (primer CYcald_F, CY_R)*; 4: *POP2 (primer CM_F, CM_R)*; 5: *SPORES (primer GM_F, GM_R)*; 6: *SPORES (primer GS_F, GS_R)*; 7: *SPORES (primer CYcald_F, CY_R)*; 8: *SPORES (primer CM_F, CM_R)*; 9: Negative control).

Discussion

Biofilm growing on building materials are usually retrieved as multispecies aggregations in which the presence of several ubiquitous phototrophs and heterotrophs leads to self-sufficient complex consortia (Tomaselli et al., 2000), able to resist to the fluctuating conditions of rock-atmosphere interface (Zakharova et al., 2013).

When the environmental conditions are harsh and prohibitive as in the volcanic environments, only few species are able to successfully colonize the rocks (Rothschild and Mancinelli, 2001). In our survey at the Stufe of Solfatara, the identified microrganisms were all unicellular autotrophs belonging to the Order of Cyanidiales

(Rhodophyta). Despite their simple morphological features they display a large diversity (Ciniglia et al., 2004) hence requiring great efforts for the isolation of axenic species. Flow cytometric analysis and sorting allowed to easily recognize and isolate the genera *Cyanidium* and *Galdieria*, especially on their cellular size and cell complexity features. Morphological features that can hardly be attributed with optical microscopy even at high magnification become relevant when analyzed through FACS systems. Our findings were then also confirmed with the use of novel designed species-specific primers. Specific molecular variability of plastidial gene rbcL allowed to conceive a fast PCR assay in which purity and the identity of Cyanidiales strains can be asserted.

Conclusion

The present study offers a useful tool for the identification and the maintenance of Cyanidiales strains, from both environmental and culture collection origin. The simple and fast application of flow cytometry and PCR techniques not only permits the identification of these microrganisms but also the isolation of the small *C. caldarium*. Moreover, the use of flow cytometry for the detection of phototrophic components in subaerial biofilms may lead to significant progress in the study of biodeterioration itself. Non-filamentous aeroterrestrial microalgae can be analyzed with flow-cytometry and sorted, allowing to skip the time-consuming cultivation and isolation process.

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CHAPTER 6

In vitro *colonization experiments for the assessment of mycelial growth on a tuff substratum by a* Fusarium solani *strain isolated from the Oplontis (Naples, Italy) archaeological site*

INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 8, Issue 4, October-December 2017: XXX-XXX www.ijcs.uaic.ro

IN VITRO COLONIZATION EXPERIMENTS FOR THE ASSESSMENT OF MYCELIAL GROWTH ON A TUFF SUBSTRATUM BY A *FUSARIUM SOLANI* STRAIN ISOLATED FROM THE OPLONTIS (NAPLES, ITALY) ARCHAEOLOGICAL SITE

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Abstract

In order to investigate the mycelial structure of rock-inhabiting fungi, an *in vitro* colonization test has been set with a low carbon source supply. A surface overlay documentation of the spreading colonies and their hyphal branching was observed both by metallurgical microscopy and fluorescence microscopy with the use of a fluorescent chitin and cellulose binding dye, calcofluor, during the whole experiment. The thickness of the fungal mat was also measured in central, medial and distal areas of the colony for each tuff tile, using a metallurgical microscope. Finally, after 20 days the tiles were also observed with CLS-microscope and all the photographic documentation was used for a segmentation image analysis on Fiji software to calculate the overlay and the volume of the mycelium. Our findings confirm that *in vitro* experiments coupled with microscopic observations are useful tools to evaluate and quantify fungal biomass on a stone substratum, especially in the early steps of fungal colonization.

Keywords*: Fusarium solani, colonization of hyphae, primary bioreceptivity, image analysis, CLSM confocal microscopy*

Introduction

Fungi can be a serious threat for cultural heritage and artworks and are among the major agents of microbial deterioration of building stones [1] since they can establish on monuments made of different lithic materials, including granite, limestone, marble, sandstone [2]-[4]. Climatic conditions may be harsh and may also not allow rapid growth of mycelia; nevertheless, fungi can develop a low profile growth, forming small colonies or unicellular aggregates with a high surface to volume ratio that allows them to thrive also under the limiting and fluctuating conditions of open environments [5], [6]. Dispersal of fungi is achieved through the formation and propagation of spores which can rest on bare substrates, also in the presence of very reduced organic sources, thanks to loose structure colonies at the microbe - mineral surface that helps the cells to adhere to the substratum and to assimilate nutrients [7]. In this respect, under the reduced availability of nutrients typical of lithic materials the first organism that colonize a virgin substratum will gain a primary advantage [8].

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Fungal biofilms accelerate the weathering processes of lithic materials by enhancing the irregularities of the surface (ridges and groove), penetrating inside the pores of the stone [9], and also constructing new ducts and cavities into intact mineral material [10].

In a survey conducted on the Roman monuments of Oplontis, Italy, we have observed a frequent occurrence of patinas caused by fungal growth on tuff walls: the following efforts to isolate these organisms led to the identification, among other less frequent genera, of fungal strains belonging to *Fusarium solani*. *Fusarium* is a genus of filamentous fungi (Hypocreales, Ascomycota,) commonly retrieved as soil contaminant and plant pathogen [11]; but also frequently found as a component of biofilms deteriorating stone monuments [12]-[14].

The understanding of the early steps of microbial colonization is a challenging issue for the biology of biofilms. Complicate interactions occur in biofilms, which involve any possible microbial individual in the formation of communities that are able to persist and flourish in extremely variable and harsh environments such as rocks and building materials. Though fungi appear to behave as a late colonizer of biofilm, in the opportune environmental conditions they can also act as pioneers, starting wide patinas that lead to the discoloration and the alteration of the material [15] [16].

Here we present an *in vitro* laboratory test coupled with optical, fluorescence and confocal microscopy, to study the early steps of the colonization of yellow tuff tiles by a *Fusarium solani* strain (ACUF 016f) isolated from the archaeological site of Oplontis. Literature is poor as regards the assessment of fungal growth on stone substrate [17] [18]; in most cases, the inoculum of the fungus is not reproducible or the initial medium supply is composed of more than one carbon compound, giving misleading results in the following observations. One of the issues linked to the use of small inoculum and limiting carbon source, is the subsequent way of determining the biomass and the colonization degree of the colony. Hyphae can be thin and translucent for non-melanized fungi, and difficult to detect on a matt substratum [19].

Few studies have been performed on thickness variability of biofilms growing on hard substrates [20][21][22], with the aim of providing a quantitative basis for analysis of microscale architecture of biofilms[23][24], especially for the investigation of bacterial populations [25]. In order to investigate the mycelial structure of rock-inhabiting fungi, the growth of F. solani on yellow Neapolitan tuff tiles has been followed in sealed glass chambers under low sucrose concentration and high Relative Humidity (RH). This *in vitro* model allowed us to shed light on bi and three-dimensional evolution of the fungal mycelium by using a polyphasic approach based on the use of epifluorescence and metallurgical microscopy in bright field combined with CLS-microscopy.

Material and Methods

Sampling site description and identification of a Fusarium solani *strain*

The strain used for this experiment has been collected in a survey at the Oplontis archaeological site (Fig. 1, top and left bottom). Sample was collected from the external tuff walls of the residential villa, known as the "Villa of Poppea" with sterile scalpels, and grown in the lab on PDM agarized medium. Fungal hyphae were isolated with the aid of a stereomicroscope and then separately cultivated, using the same culture medium. The identification of a *F. solani* strain (Fig. 1, right bottom) was initially assessed on the basis of morphological observations, and confirmed by molecular analysis. DNA was extracted with a modified Doyle and Doyle DNA extraction protocol [26] and used for a Polymerase Chain Reaction with primers targeting ITS spacers (primer forward 5'-TCCGTAGGTGAACCTGCGG-3'; primer reverse 5'-TTCAAAGATTCGATGATTCAC-3'). PCR was carried out in a 25 µl aliquot containing approximately 100ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x

buffer), supplemented to give a final concentration of 2.5 mM MgCl2, 1.25U of Taq polymerase (EconoTaq, Lucigen), and 0.5 pmol of each primer. Amplification was run in an Applied Biosystem 2720 thermal cycler. The profile used was 5 min at 95°C, 33 cycles of 95°C for 30s, 60°C for 45s, and 72°C 45s, and a final elongation step of 7 min at 72°C.

Fig. 1. Detail of the archaeological site of Oplontis (top), detail of the biofilm on the surface of a tuff wall (bottom left) from which it was isolated *F. solani* (bottom right).

PCR product was evaluated on 1.4% (w/v) agarose gel in an electrophoretic run and purified using QIAquick® PCR Purification kit (Qiagen Inc, Valencia, CA, USA). Sequence reaction was obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), purified in automation using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA 01915 - USA) and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA). The product was analyzed by an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The amplification primers were used as the sequencing primers.

Nucleotide sequence similarity was determined by using BLAST version 2.0 (National Center for Biotechnology Information databases). The isolated strain of *F. solani* was maintained following the protocol by McGinnis et al. [27].

Roughness and Porosity analysis, Petrographic data

In many archeological sites the use of local stone for architectural purposes very frequent; in Campania region (Italy) the large availability of volcanic products as Neapolitan Yellow Tuff (NYT) and their easy workability, determined their great utilization since the earliest times [28]**.** The lithic samples used in in vitro tests were taken from the caves Neapolitan Yellow Tuff (NYT) of Quarto (Napoli). NYT samples were cut especially with measures suitable for the needs of this experiment (average size 3x3x1 cm).

According to standard ISO 4287:1997 [29] the roughness parameters were evaluated on each sample with an ALPA© RT-20 palmar rugosimeter. All measurements were performed in triplicate, and data acquisition was conducted using the Measurement Studio Lite 1.0.3.96

software. The porosity of tuff lithic samples was assessed through mercury porosimetry (Autopore 4, Micromeritics®). The water absorption coefficient (WAC) of the tiles (mean ± sd) was calculated according to Barberousse et al. [30]. Eight lithic samples were held at a constant temperature (21°C) and relative humidity (51-55%) for two weeks. The density (mean ± sd) of the tuff was obtained by evaluating the ratio weight/volume.

Laboratory strains and Culturing conditions

All the tuff tiles used in the experiments were washed with sterile water, dried and displaced in triplicate in glass chambers, which were tyndallized. The tiles were then watered at their maximum absorbance capacity with sterile Bold's Basal Medium (BBM) [31] added with sucrose 12g/L, according to Jeger et al. [32].

F. solani conidia were obtained from a 5 days old colony treated for 1 minute with PBS-Tween20 solution at the final concentration of 0,5% and mechanically scraped with a sterile handle. The suspension was then recovered and filtered through a sterile gauze, conveniently diluted and the number of conidia per milliliter was determined through a direct microscopic count in a Bürker blood-counting chamber. In all the experiments the inoculum consisted of 5000 conidia suspended in 5µl of sterile distilled water, injected with a pipette tip in the middle of each tile. During the whole time of observation, no bacterial contamination was found. One more glass chamber was prepared with tuff tiles watered with distilled water instead of nutritive medium and kept until the end of the experiment as a control.

To evaluate the fungal growth, every 4 days three tuff tiles from each of the three glass chambers were analyzed, and once the measures were taken they were discarded.

F. solani ***growth under different sucrose concentrations or relative air humidities***

Preliminary experiments were carried out to evaluate the kinetic of *F. solani* fungal growth according to Table (1). Six different concentrations of sucrose in the medium were used, ranging from 1.5 g/L to 48 g/L, at a constant RH of 100%. The fungal overlay was then assessed by digital image analysis. The relationship between relative humidity and *F. solani* growth on tuff tiles was assessed according to Häubner et al. [33]. In the bottom of four glass chambers were poured distilled water or saturated salt solutions of NaCl, KNO_3, $Ca(NO_3)_2$, to provide respectively 100, 93, 85, 76%, relative air humidity; the tiles inoculated with the fungal spores were positioned on perforated ceramic grilles placed at about two cm from the solutions, and each glass chamber was covered with a glass lid and sealed with silicone foam.

Both RH and sucrose experiments were monitored for 12 days: every 4 days, 3 samples were photographed with a digital camera. Digital image analysis was applied to quantify the growth of the mycelium on the stone samples inoculated at different sucrose concentration or at different values of Relative Humidity. The photographic recording of each tile was performed at 3 incubation times (4, 8 and 12 days) with a digital camera (Nikon D5100 with Nikkor 50mm objective). The conventional RGB color images obtained with the digital camera were used to quantify the colonization area on each lithic sample with the program *Trainable Weka Segmentation* [34], [35], a plugin of open source image processing package *Fiji*, an open source image processing package [36] and also at http://www.fiji.sc.

F. solani ***growth in constant nutrients and moisture***

The methods previously described were used to select the best experimental conditions to be adopted in the following test, planned to describe the mycelial structure of *F. solani* biofilm on tuff tiles.

The tests were performed at 12 g/l sucrose and at 100% RH and lasted three weeks. In this set of experiments, ten points of observation were chosen for each tile, as previously described, and the observations were carried out at an interval of four days.

For the assessment of the fungal mat architecture on the lithic substrate, five glass chambers were prepared for the test, under the same experimental conditions. Fifteen tuff tiles were inoculated with *F. solani* conidia and also distributed in the 5 glass chambers at 100% RH as previously described. The experiment lasted three weeks and was repeated two times.

Each tile was virtually divided into three zones ranging from the middle to the external borders of the tile and 10 points of observation, 2 central (near the inoculum), 4 median (average distance of 9 mm from the center of the sample) and 4 distal (average distance of 18 mm from the center of the sample) were selected (Fig. 2, left). To evaluate the fungal growth, the experiment was monitored for 20 days: every 4 days, three tuff tiles from each of the five glass chambers were analyzed, and once the measures were taken they were discarded**.**

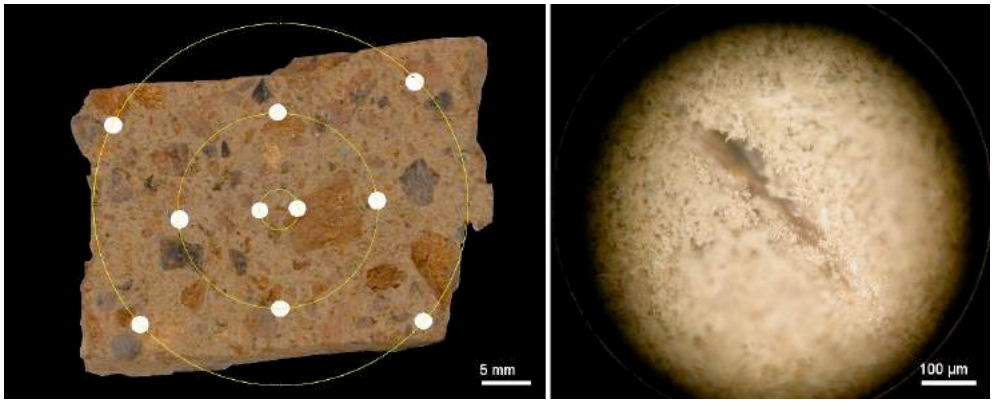

Fig. 2. Representation of the 10 measuring points of the biofilms of *F. solani* on the tuff tile (left); an example of biofilm cutting for thickness calculation (right), metallurgical microscope photos.

Each set of measurements was performed for three weeks at an interval of 4 days in the following way:

1. Quantification of the colonization area. The samples of tuff tiles were photographed with a digital camera. From the digitized images, the coverage area was calculated by means of WEKA segmentation showing the colonized areas of the tile, and by using Analyze Particle.
2. Measurement of the fungal thickness. In the 10 selected points, the hyphal network was cut with a pen cutter (Fig. 2, right) equipped with blade oblique (WLXY®, model number: WL9309). According to Bakke and Olsson [37] the thickness values were determined with a metallurgical microscope (Leitz Wetzlar Ortholux Microscope) with an objective 4x.
3. Measurement of the mat surface texture. The same tiles used for the measurement of thickness were then sprayed with Calcofluor White 1% [38], a fluorescent dye that binds cellulose and chitin in the fungal wall The observations were carried out on the 10 selected points with an epifluorescence microscope (Nikon Eclipse E800) at 20x magnification, using the DAPI filter (excitation 395 to 415 nm, emission from 455 nm). A photographic documentation was collected and later used for computer image analysis, with the threshold tool of *Fiji* software, to evaluate the coverage area of the hyphae network.

The results obtained by the triplicates for any given set of measurements for both the thickness and surface data were used as means for each observation point and then plotted with their respective standard errors.

At the end of the experiments (20^{th} day), the growth of *F. solani* on three tuff tiles was also analyzed with a Confocal Microscope Zeiss LSM700 (software Zen 2011) by capturing

images at 10x. The fluorescence of hyphae was recorded in one channel using the Calcofluor white to label the hyphae with excitation beams at 405-458 nm and emission at 415-505 nm (blue-green channel). The images from stacks were captured at 13.46 μm intervals. For each biofilm, 3 replicates were used for taking Z-stacks images. The substratum area of the image of the epilithic stack was 1024 x 1024 pixel (640.174 x 640.174 μm). The number of images in each stack varied according to the thickness of the biofilm. Fiji was also used to evaluate the area of the fluorescence photographs and all stacked CLSM images. The images have been previously converted to 8-bit and then resampled by using the tool Threshold [39]-[41]. *Comstat2* [42], *3D Manager* [43] and *DiameterJ* [44] tools were used to determine the volume, thickness, roughness and features of the hyphae of each Z-stacks [45].

Table 1. Number of tiles (in triplicates) and sucrose and RH conditions for each set of experiments.

	Tuff tiles	**RH** (%)	**Sucrose concentration** (g/L)	**Observation time** (1t = 4 days)	**Duration** (days)
Different RH	9	76 85 93 100	12	3	12
Different sucrose	9	100	1.5 3 6 12 24 48	3	12
Constant RH and sucrose	15	100	12	5	20

Results and Discussion

Roughness and Porosity analysis, Petrographic data

The NYT shows its typical assemblage of prevailing epigenetic phases (phillipsite, chabazite and analcime), feldspar, and a minor amount of mica, hydrated iron oxides and volcanic glass [46]. The roughness profile was calculated considering 1600 sample points for each lithic sample to determine the average roughness (Ra = 19.32), the root mean square surface roughness (Rq = 23.21), mean roughness depth (Rz = 85.68) and maximum or total roughness (Rt = 101.83). The porosity parameters of tuff lithic samples were calculated, along with density and water adsorption coefficient (Table 2).

Table 2. Water adsorption coefficient (WAC), porosity and roughness of tuff tile.

WAC ($g\ dm^{-2}\ min^{-1/2}$)	Porosity				Roughness			
	Density ($g\ cm^{-3}$)	Porosity (%)	Average pore diameter (nm)	Total pore area (m^2/g)	Ra	Rq	Rz	Rt
49.28^{-5}	1.461	56.63	247.8	9.47	19.32	23.21	85.68	101.83

Preliminary assessment of* F. solani *growth under different sucrose concentrations or relative air humidities

A development of fungal hyphae on tuff was observed since the first week of incubation, as reported for other accelerated test of stone colonization by fungi [47].

At all the selected sucrose concentrations a *F. solani* fungal mat developed on the surface of the tiles, and a linear increase of surface coverage was observed between 1.5 and 24 g/L of sucrose concentration (Fig. 3, left). At 24 and 48 g/L sucrose, the surface of the tiles was completely covered by *F. solani* hyphae at the end of the experiments. According to experimental data of Chertov et al. [48] and the mathematical model developed by Picioreanu et

al. [49], the spread of *F. solani* depended on nutrient concentration, and sucrose concentration lower than 24 g/L that did not allow the complete coverage of the tiles during the course of the tests were considered as limiting.

The effect of the atmospheric relative humidity on *F. solani* growth was assessed in the range 76-100% RH. In these experiments, a sucrose concentration of 12 g/L was used to supplement the inorganic culture medium (Fig. 3, right). The results of digital image analysis show that HR levels from 82 to 100% equally supported the growth of *F. solani* during the whole experiment. It is known that microorganisms can survive also under very reduced water availability, but in that case, they are not able to grow [49]. *F. solani* growth is dependent on relative humidity (Fig. 4) but, contrary to what reported by Dubey and Jain [50] fungal growth on the tuff stone was observed also at 76% RH, probably due to the high water adsorption coefficient of tuff stone and its ability to retain moisture.

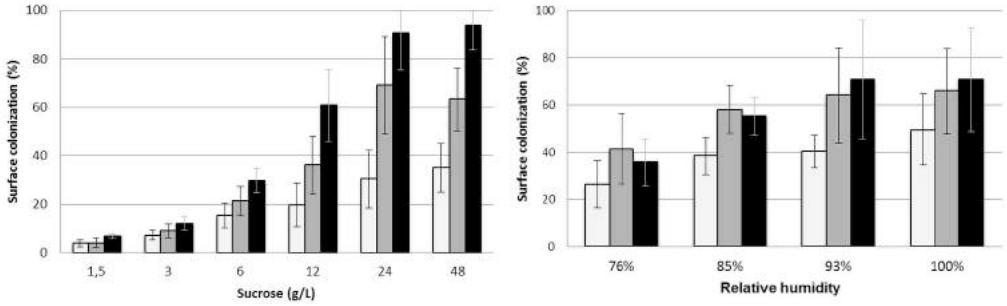

Fig. 3. Growth (percentage coverage) of *F. solani* on tufa tiles: different concentrations of nutrients and at 100% RH (left), different 100% RH and to sucrose concentration of 12 g/L (right) at 4 (light grey), 8 (dark grey) and 12 (black) days incubation.

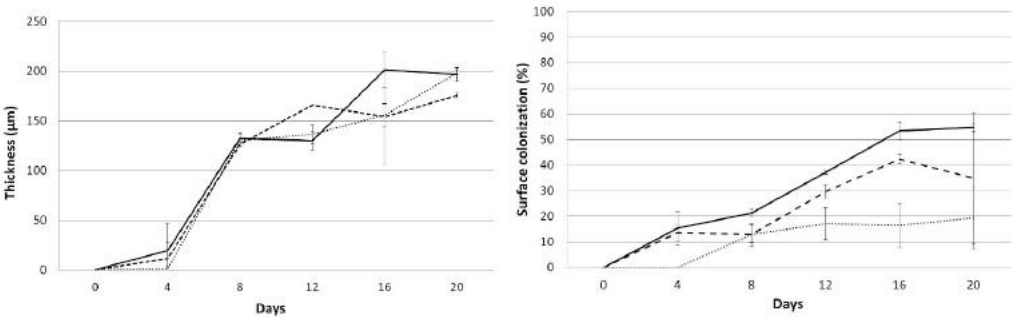

Fig. 4. Percentage of thickness of biofilm growth on tufa tiles (left), surface colonization on tufa tiles (right); points: central (——), median (- - -), distal (· · ·).

F. solani *mat structure under constant nutrients and moisture*

The growth of hyphal filaments was observed in all the observation points corresponding to central, median and distal regions of the tiles (Fig. 4, left). In the central region, the aerial mycelium increases its thickness during the first 15 days of incubation, reaching a plateau at the 16th day. The maximum average thickness appears to be fixed to about 200 µm for all the considered regions on the tile. A similar pattern, albeit shifted in the time, was also observed in the median region, where the plateau was reached at the 20th day, whereas in the distal region *F. solani* growth was significantly lower, and the hyphae were unable to colonize all available space in the course of the experiments, due to limiting available nutrients (Fig. 4, right). In this region a noteworthy development of conidiophores was observed (*not shown*).

Fungal growth on tuff tiles results in a mycelium with a reticulate texture, with open spaces that separate the fungal mat, leading to a patchy structure of the fungal growth. The development of a reticulate texture could be due to change in the growth direction of filaments, as reported for cyanobacterial growth on lithic substrates [51]. The surface texture of the hyphae (sprayed with fluorescent brightener Calcofluor White) during the colonization of the substrate was observed with an epifluorescence microscope. Data show that in each region of the tile there is a constant increase of the hyphal branching over the time, that are leading to a progressive reduction of the void dimension in the texture.

Surface texture and thickness are not related (R^2 = 0.45), suggesting that in our experimental conditions the hyphal organization is oriented toward a higher superficial branching degree more than increasing the thickness of the mycelium.

In order to assess if the spatial organization of hyphal branching is subject to major changes along the layers forming the mat, and/or in the three regions of the tile, we performed a three-dimensional evaluation of the hyphal branching by CLS-microscopy (Fig. 5). At the end of the 20th day of incubation, the tuff tiles were sprayed with Calcofluor white and then analyzed with a confocal microscope in order to evaluate some structural parameters in the central, medial and distal region of the biofilm (Table 3, top). Z-Stacks analysis has shown a clear resemblance of structural patterns of hyphae in the central and median regions. To evaluate the characteristics of the hyphae network, we analyzed z-stack MIP images with Fiji's *DiameterJ* plugin (Table 3, bottom).

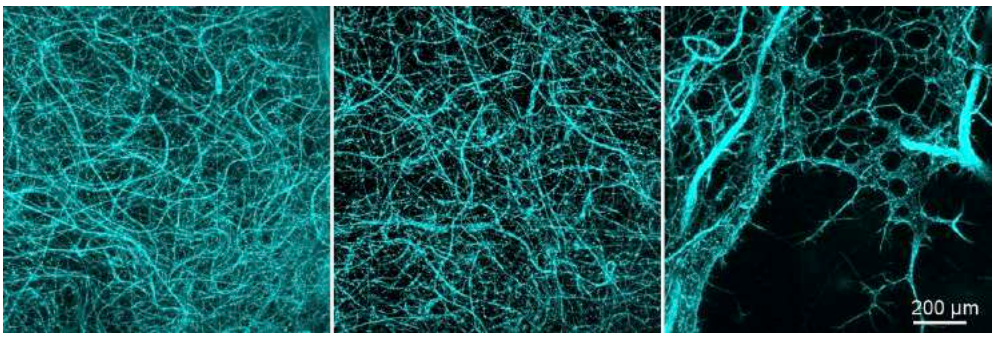

Fig. 5. MIPs of *F. solani* biofilm on lithic tuff tile, central (left), median (central) and distal (right) z-stack to the biofilm growth surface.

Table 3. Analysis of various architectural parameters of *F. solani* biofilm

	Central	Median	Distal
Area MIP (%)	44.112±1.822	35.941±3.862	11.878±0.665
Biomass (µm^3)	32780434.778±1051337.997	33470831.368±2373961.800	20381191.392±19922166.572
Thikness average	215.7985±113.608	220.940±3.080	125.140±64.875
Thikness max	420.000±141.421	334.500±11.313	240.000±133.643
Ra	0.7355±0.057	0.645±0.041	1.417±0.286
Mean pore area (µm^2)	28.514±5.499	30.497±3.156	34.803±8.2654
Max. pore area (µm^2)	1241.723±614.373	2654.363±258.238	3734.674±1821.298
Number of pores	719±226.981	964±151.228	270±98.558
n° of intersections	31213±620.386	50680±1380.868	14984±1569.448
Diameter mean (µm)	1.176±0.215	1.87±0.561	4.867±1.256
Diameter max (µm)	4.575±2.121	10.457±4.85	68.071±10.684

According to the consideration of Matsuura and Miyazima [52], that larger leading hyphae continue to extend, whereas secondary hyphae seem to be more sensitive to nutrient depleting conditions, we observed a prevalent presence of very thick hyphae in the distal region of the tuff tiles.

The three-dimensional architecture of fungal biofilm on different substrates is largely influenced by the environment: mat formation by *Saccharomyces cerevisiae* was grown on medium containing a low agar concentration showed a radial pattern of growth, with a central hub made of a complex network of cells and radial spokes, originated from the hub. This structure was influenced by the viscosity of the medium, and nutrient availability [53]. Similarly, CSLM analyses performed on *Fusarium* populations grown on soft contact lens revealed that biofilm thickness was limited by the water content of lenses and that the architecture of fungal mat was dependent on surface characteristic of lenses, albeit remaining uniform at the center and periphery [53].

In our experiments, the structural features of yellow Neapolitan tuff seem to dictate a different three-dimensional structure of *Fusarium solani* mats. Table 3 shows that the roughness of the mat increases from central to distal regions of the tiles, these latter showing an almost double roughness compared to the central and median regions. Moreover, there is an increase in the average and maximum size of the hyphae network voids from the central to the distal region of the tile, and also the values of the mean and maximum diameters of the hyphae show an increase from central towards distal regions of the tile. Finally, the number of intersections of hyphae has a drastic decline from the center to the borders of the tile (Table 3). All these results concur to indicate that the mycelium in the central and median regions is compact and consists of a very intricate network of prevalently thin filaments, and that is homogeneous along the stacks. Conversely, in the distal areas, the mycelial network is loose and double-stranded filaments prevail. Also, in this case, no vertical zonation of hyphal architecture was evidenced.

Conclusions

In vitro colonization experiments can be useful to understand the role of environmental parameters such as relative humidity, temperature, light intensity and nutrients on the fungal three-dimensional organization. Our tests have indicated that *F. solani* is able to colonize the tiles also in very limiting conditions of carbon supply and relative humidity and that the yellow Neapolitan tuff is a very bioreceptive material. Using the in vitro model, we showed that the colonization of the tuff tiles exhibited a well-defined pattern:

- The surface roughness of the biofilm and mean and maximum diameters of the hyphae showed an increase from central towards distal regions;
- the number of intersections of fungal filaments declined from the center to the borders of the mat, causing the increase of the average and maximum size of the voids in the distal region of the mycelium.

A combination of microscopical techniques (epifluorescence, metallurgical microscopy in bright field and CLS-microscopy) can be successfully applied to assess the first steps of fungal colonization on stone substrate, especially to evaluate thickness and structural texture of the mycelium, less investigated parameters in biodeterioration studies although they play a key role in the establishment of mature biofilms pioneered by fungi.

Acknowledgments

The authors thank the Parco Archeologico di Pompei, and particularly Dr. De Carolis for the kind guidance and support. The authors gratefully acknowledge to Benedetto De Fazio for his valuable technical support.

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Received: February 16, 2017
Accepted: November 20, 2017

CHAPTER 7

A biological and quantitative study on in vitro colonization of Neapolitan yellow tuff by Alternaria tenuissima

A biological and quantitative study on *in vitro* colonization of Neapolitan yellow tuff by *Alternaria tenuissima*

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Abstract An *in vitro* colonization experiment has been performed to investigate epilithic and cryptoendolithic growth of the fungus *Alternaria tenuissima*. Colonization test proceeded for 20 days on tuff stone with low carbon source supply. Growth has been documented for the whole experiment duration with photographical documentation of the spreading colonies, monitored with both metallurgical microscopy and CLSM microscopy, with the use of a fluorescent chitin-binding dye. Thickness of the biofilm was also measured in central, medial and distal areas of the colony for each tuff tile. Moreover, overall growth af the fungus has been measured with total conidia count, ergosterol determination through HPLC and total DNA extraction. Our findings confirm that in vitro experiments coupled with microscopical observations and computer image analysis are useful tools to evaluate and quantify fungal biomass on a stone substratum, especially in the early steps of fungal colonization.

Keywords: *crypto-endolithic growth, weathering, image analysis, CSLM confocal microscopy*

Introduction

Fungi can be a serious threat for cultural heritage and artworks and are among major agents of microbial deterioration of building stones [1]. Besides the spoiling due to color change and patina formation, they can deeply colonize cracks and fissurations because of the extraordinary penetrating power of their hyphae into the substratum, causing breakings and lesions to the artwork.

Harsh environmental conditions and wide fluctuations of relative humidity, light exposure, available nutrients and temperature are limiting factors for microrganisms that thrive on stones; nevertheless, fungi are able to tolerate these adversities keeping their viability on building materials also under the 50% of relative humidity [2].

Tuff has been a very common building material in the area of Pompeii over the centuries [1]. Particularly, Neapolitan yellow tuff is incredibly porous, with a considerably high water absorption coefficient [3] and its pores act as perfect niches for the colonization of microrganisms.

A better understanding of biodeterioration mechanisms and its effects on materials are needed in order to preserve constructions from fungal colonisation; to this aim, in vitro

experiments are useful to assess bioreceptivity of different lithotypes [4] and biodeterioration due to phototrophs colonisation [5] or fungal colonisation [6].

In our previous surveys on biofilms sampled from the archaeological sites of Herculaneum, Pompeii and Cumae (Campania, Italy), the presence of *Alternaria* was confirmed to be costant amongst the identified molds. *Alternaria* is a genus of filamentous fungi (Ascomycota, Pleosporales) commonly retrieved as soil contaminant and plant pathogen; most of the alternarioid species are considered to be cosmopolitan saprobes that are ubiquitous through natural and manmade environments [1]. *Alternaria* is also frequently found in the biofilm deteriorating stone monuments [2-5].

Here we present a laboratory test to study the initial steps of fungal colonization above tuff tiles. An *Alternaria* strain sampled in the portico of the southern garden (Oplontis) was chosen, due to the peculiar shape of its conidia, of the melanized hyphae and its easy cultivation. Fungal growth has been followed in a 20 days experiment in which a a controlled environment was created with a single carbon source and high relative humidity in glass petri dishes containing yellow tuff tiles. Several typical parameters of the fungal growth have been recorded each 4 days, such as number of conidia, thickness of the fungal mat and biomass estimation through total DNA extraction and ergosterol quantification.

Since the peculiar features of tuff substratum, we focused on characterizing the architecture of a fungal mat both on the surface of the stone and inside its cracks and fissurations. A surface overlay documentation of the spreading colonies and their hyphal branching observed with metallurgical microscope was taken during the whole experiment to assess mycelial architecture and its development on a stone substratum. Also a description of hyphal penetration was provided through CLSM-microscopy stack reconstruction in order to evaluate crypto-endolithic growth of the fungus.

Materials and Methods

Petrographic analysis, roughness and porosity

In many archeological sites the use of local stone for architectural purposes has been a very wide common. In Campania region (Italy) the large availability of volcanic products and their easy workability, determined the their great utilization. The lithic samples used in in vitro tests were taken from the caves Neapolitan Yellow Tuff (NYT) of Quarto

(Napoli). The NYT shows its typical assemblage with prevaling epigenetic phases (phillipsite, chabazite and analcime), feldspar, and minor amount of mica, hydrated iron oxides and volcanic glass [7]. According to standard ISO 4287:1984 [8] the roughness parameters were evaluated on each *tessera* with an ALPA© RT-20 palmar rugosimeter. All measurements were performed in triplicate, and data acquisition was conducted using the Measurement Studio Lite 1.0.3.96 software. We calculated the roughness profile from 1600 sample points for each tile to determine the average roughness (Ra), the root mean square surface roughness (Rq), mean roughness depth (Rz) and maximum or total roughness (Rt). We report the above data with the mean density and the water absorption coefficient only for the tiles for which we obtained meaningful microbial growth. The porosity of tuff lithic samples was assessed through mercury porosimetry (Autopore 4, Micrometrics). Before assessing the density and water adsorption coefficient, eight lithic samples were held at a constant temperature (21°C) and relative humidity (51-55%) for two weeks. The density (mean ± sd) of the tuff was obtained by evaluating the ratio weight/volume. The water absorption coefficient (WAC) of the tesserae (mean ± sd) was calculated according to Barberousse et al. [9].

Origin and culture of the strains

The strain used for this experiment has been collected in a survey at the portico of the southern garden in Oplontis. Together with the other components of the biofilm, it was identified through a polyphasic approach. Samples were cultivated on agar PDB (BD Difco™ Potato Dextrose Broth, USA), isolated with the stereomicroscope and then observed with the optical microscope. A further confirmation of the observations and the assessment of the species was obtained through a molecular analysis.

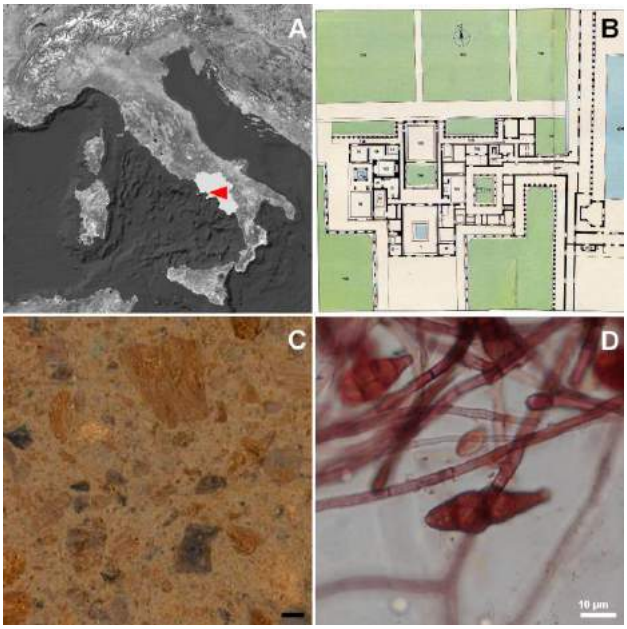

Fig. 1. Location of the archaeological site of Oplontis in the Campanian region (A), surface detail of a tuff a wall (B) from which it was isolated *A. tenuissima,* neutral red staining, 40x magnification (C).

The isolated strains were kept in the ACUF collection (Algal Collection of University Federico II) of Department of Biology in Napoli, Italy following the protocol by McGinnis et al. [10]. Nutritive medium used for biodeterioration test was composed of BBM (Bold's Basal Medium) [11] plus the addition of sucrose 12g/L, according to Jeger et al. [12].

DNA-based molecular analysis

DNA was extracted with a modified Doyle and Doyle DNA extraction protocol [13] and used for a Polymerase Chain Reaction with primers targeting ITS spacers (primer forward 5'-TCCGTAGGTGAACCTGCGG-3'; primer reverse 5'-TTCAAAGATTCGATGATTCAC-3'). The amplification product was then evaluated by agarose gel electrophoresis, purified with QIAquick® PCR Purification kit (Qiagen Inc, Valencia, CA, USA). Sequence reaction was obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), purified in automation using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA 01915 - USA) and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA). Product was analyzed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The amplification primers were used as the sequencing primers. The

obtained sequence was loaded in BLAST version 2.0 (National Center for Biotechnology Information databases) and identified as *Alternaria tenuissima*.

Accelerated colonization

The Neapolitan Yellow Tuff tiles (average size 3 x 3 x 1cm) were displaced in triplicate and tyndallized in 5 glass petri dishes, one for each experimental observation. The tiles were then watered at their maximum absorbance capacity with the nutritive medium while preserving sterility. The inoculum consisted of 5000 conidia suspended in 0.9% of NaCl solution and injected in the middle of the tile. Conidia were obtained from a 5 days old *A. tenuissima* colony treated for 1 minute with PBS-Tween20 solution at the final concentration of 0.5% and mechanically scraped with a sterile handle. The suspension was then recovered and filtered through a sterile gauze, conveniently diluted and number of conidia per milliliter was determined through a direct microscopic count in a counting chamber (Burker blood-counting chamber, HBG – German). Glass petri dishes (100 x 15 mm) containing the tuff tiles with the inoculum were kept at 100% relative humidity with sterile filter paper discs soaked in sterile distilled water and stored at 26°C in a climatic cell. A petri dish missing of the inoculum was prepared in the same manner and kept for 20 days as a control.

Image analysis

Digital image analysis was applied to quantify the hyphae growth on and into the stone samples after 4 incubation time. The photographic recording of each *tessera* was performed with a digital camera (Nikon D5100 with Nikkor 50mm objective). The conventional RGB color images obtained with the digital camera were used to quantify the colonization area on each lithic sample. The program *Trainable Weka Segmentation* [14, 15], a plugin of open source image processing package *Fiji* [16, and also http://www.fiji.sc], was used to evaluate the colonized areas of each photograph (Fig. 2A).

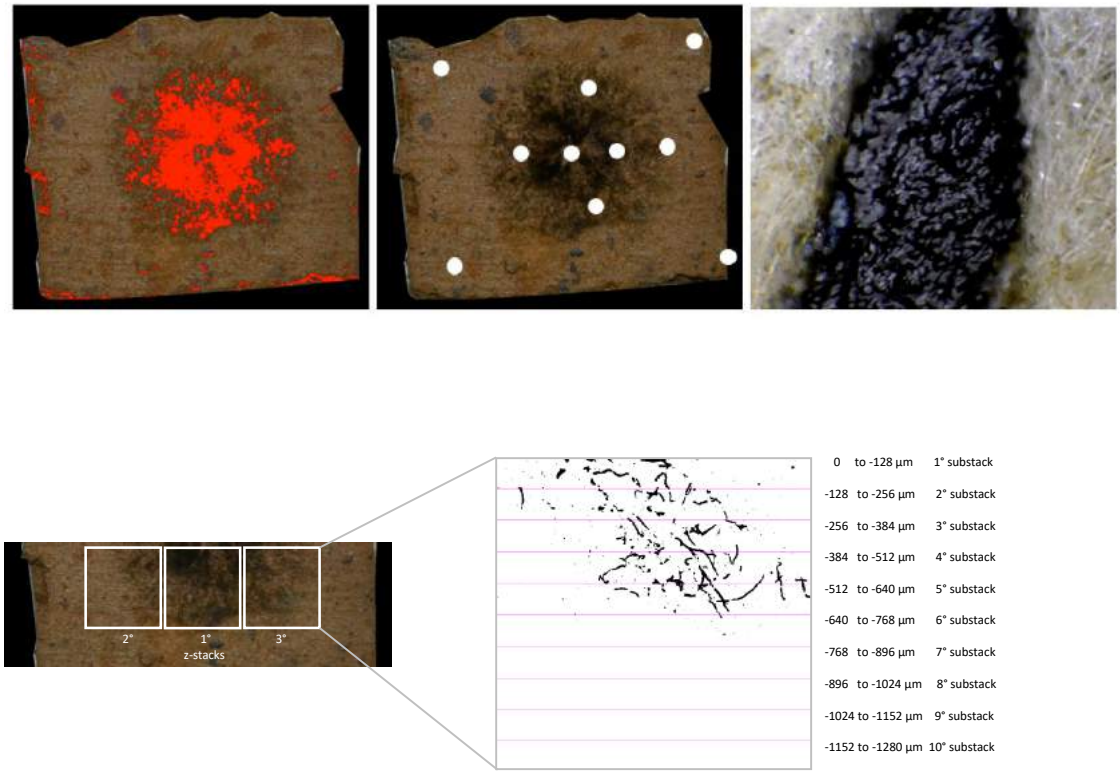

Fig. 2. A. Segmentation analysis for the determination of surface overlay of fungal colony; B. ten observation points for each analyzed tile were used for the measurement of thickness and local fungal overlay with metallurgical microscope. The observation points were then classified as central (2), median (4) and distal (4) region. C. Pen cutter blade cut in aerial mycelium for the determination of thickness with metallurgical microscope. D. Observation points for CLSM analysis and substacks division for crypto-endolithical determination of hyphal growth.

Optical, metallurgical and laser confocal microscopy (CLSM)

Optical

The colonized surface of the tuff tile was energetically scraped with a razor blade and the fungal biomass was recovered into 1,5mL tubes, and suspended in 1mL 0.9% of NaCl solution. The sample was then properly diluted and aliquoted into two tubes. Conidia from each tube were counted in triplicate through a direct microscopic (Nikon Eclipse E800) count in a Burker chamber.

Metallurgical

In order to monitor fungal growth during test, the surface of each specimen was observed each 4 days with metallurgical microscope (Leitz Wetzlar Ortholux Microscope) with an objective 4x. The measurement was performed in 10 points of each tile, 2 central (near the inoculum), 4 mean and 4 peripheral (Fig. 2B). In each of 10 points were cut the

hyphae network with a pen cutter with blade oblique (WLXY®, model number: WL9309). According to Bakke and Olsson [17] the thickness values of mycelium were determine. Each observed point was also shoot with (Nikon D5100) photographical documentation was later used for computer image analysis to evaluate the covering area of the hyphae.

CLSM

The microbial populations on the substrate were also analyzed with Zeiss LSM700 (software Zen 2011) by capturing images at 10x. The fluorescence of hyphae was recorded in one channel using calcofluor white to stain the hyphae, with an excitation beams at 405-458 nm and emission at 415-505 nm (blue channel). The images from stacks were captured at 13.46 µm intervals. For each fungal mat, 3 replicates were used for taking Z-stacks images. The substratum area of the image of epilithic stack was 1024 x 1024 pixel (640.174 x 640.174 µm). The number of images in each stack varied according to the thickness of the fungal mat.

Epileptic growth was monitored through the z-stack of the top of the lithic sample. The stone samples were cut perpendicular to the inoculated surfaces (Fig. 2C) after the incubation period, no trace of hyphae was visible to the naked eye. Crypto-endolithic colonization was followed by recording to CLSM. The Z-stacks recorded of the sections perpendicular to the stone surface enabled studying the distribution of hyphae network, their development on the subsurface of the samples and their relationship with the substrata.

Fiji was used to evaluate the area of all stacked CLSM images, and to obtain 2D MIPs. The images have been previously converted to 8-bit and then resampled by using the tool Threshold [18-20]. *Comstat2* [21] tool was used to determine the volume, thickness, roughness of each Z-stacks [22, 23].

The substratum area of the image of crypto-endolitic stack was 1024 x 1024 pixel (1280.348 x 1280.348 µm). The recording to CLSM, for the hyphae crypto-endolithic growth, produced Z-stacks with parallel slices to the fracture surface (Fig. 2D). Each slice shows the hyphae nearest to the part where epileptic growth (upper part of the slices) has occurred, and even the hyphae that it grows deeper (lower part of the slices).

In order to obtain a more appropriate quantification of the network of hyphae grows crypto-endolitically, the Z-stacks have been superimposed on grids at parallel equidistant lines. This grid was used to obtain substacks, representing intervals of 128µm in depth (Fig. 2D).

Ergosterol estimation through HPLC chromatography and DNA quantification

The analysis of ergosterol content was performed as previously reported [25]. Briefly, each algae suspension was lyophilized and the crude residue in dependence of its weight was dissolved in 0.5-2.0 mL of MeOH by sonication for 3 minutes. Each suspension was refluxed for 2 hours, added with 0.3-3.0 mL of 2 N KOH in MeOH, mixed and then saponified for 30 minutes. It the end, the suspension was cooled down to room temperature and extracted with ethyl ether (10 mL), twice. The organic upper phase was analysed by HPLC Shimadzu LC-8A equipped with a Shimadzu SCL-10AVP system control and a Shimadzu SPD-10A VP UV/VIS detector, using a C18-110A prep. column (Gemini 10 mm, 250 x 21.2 mm i.d., Phenomenex) with MeOH as mobile phase. The quantification of ergosterol with the absorption maximum of 282 nm was obtained using an external standard via a five-point calibration.

Results

Lithic sample characterization

The tiles of Neapolitan Yellow Tuff used in this study were analyzed for water absorption coefficient, porosity and roughness as described in Materials and methods. Data regarding tuff characterization are consistent with the properties and description of this building material and measured values for petrographic characterization are showed in Table 1.

Table 1. Water adsorption coefficient (WAC), porosity and roughness of tuff tile.

WAC ($g\ dm^{-2}\ min^{-1/2}$)	Porosity				Roughness			
	Density ($g\ cm^{-3}$)	Porosity (%)	Average pore diameter (nm)	Total pore area (m^2/g)	Ra	Rq	Rz	Rt
49.28^{-5}	1.461	56.63	247.8	9.47	19.32	23.21	85.68	101.83

Thickness, surface overlay and viability of the colony on tuff tile

Image analysis performed on whole colony developing on tuff tiles showed a continuous overall radial growth. The areas calculated as percentage of overlay were used as means of triplicates and plotted with their respective standard errors in Fig. 3.

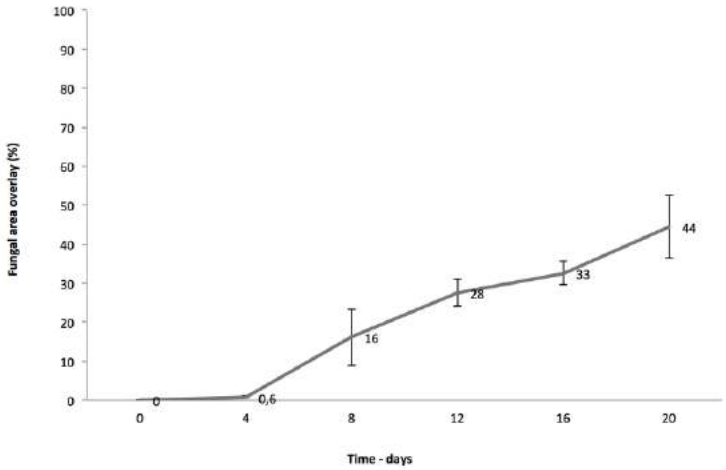

Fig. 3. *A. tenuissima* colonization on tuff tile determined with image segmentation analysis. The areas obtained for each of the triplicates were normalized to the tile surface area.

Metallurgical microscopy measurements for thickness and surface overlay were used to monitor fungal growth on the tuff tiles. Each set of measurments for the ten observation points in the tile were used in triplicate for determining the mean and the standard deviation and plotted as the proceeding of central (points 1 , 2), median (points 3, 4, 5, 6) and distal (7, 8, 9, 10) regions (Fig. 4).

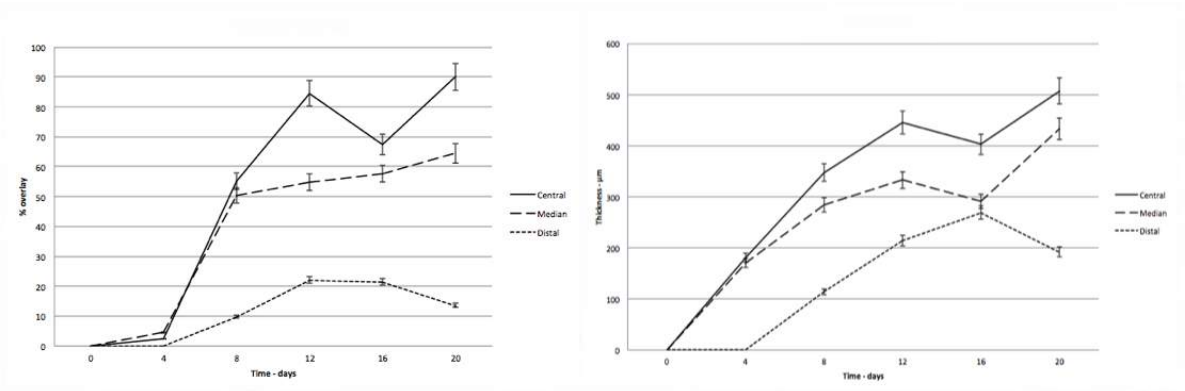

Fig. 4. Mean values obtained by metallurgical microscopy observations were plotted for each experimental time grouped as central, median and distal region for both local hyphal overlay (A) and thickness (B) of fungal colony on tuff tiles.

After 16 days incubation both thickness and overlay appearently decrease in central and medial regions, while in distal region this trend appears after 20 days incubation. We have hypothesized that both parameters may be influenced by conidia production and dispersal. Since in *A. alternata* conidia are tipically shaped as elliptical or spindle-shaped

structures their orientation toward the outside of the colony may have affected the values regarding thickness and overlay. For this reason total conidia number was also measured at the same observation times; the counts were performed in triplicate with a light microscope and a counting chamber. Also, the initial number of conidia that were inoculated on each tile may be considered negligible in the following counts, as the numeric evidence suggests that all conidia are newly formed by the colony. In the Fig. 5 it is showed that conidia production is continuous over the time in our experimental conditions and increases with an exponential trend, confirming that the inversion observed for thickness and overlay after 16 and 20 days incubation is not related with nutrients availability or an alteration state of the colony on the stone.

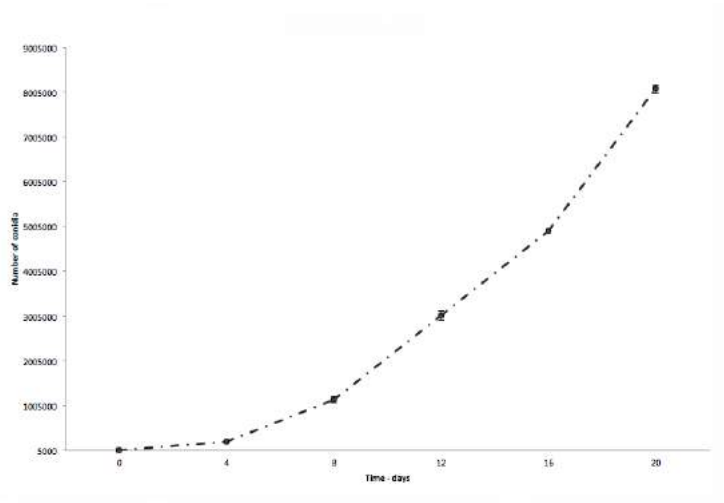

Fig. 5. Total number of conidia recorded for each experimental time.

Since the hyphal structure is not subjected to relevant movements on the substratum, it is clear that the stage of the colony and the formation of reproductive structures greatly influences the shape of the colony and its morophology also on stone substrata.

Biomass estimation through ergosterol and DNA quantification

Fungal biomass on tuff tiles was also determinated for each experimental time using both HPLC for the estimation of the total ergosterol and total DNA extraction and quantification. Ergosterol is a found in cell membranes of fungi and protozoa, where it serves the same functions of cholesterol in animal cells. It has been widely used as biomass indicator [25]. Fungal biomass has been energically scraped from the

substratum, peeling also few millimeters of substratum in which endolithic growth was supposed to be observed. The mixture of hyphae, conidia and stone debris corresponding to a quadrant of the surface, were used in duplicate for both ergosterol and DNA quantification. In Fig. 6 are showed values and correlations between ergosterol and DNA content, which have a high concordance. Both the indicators were also correlated with other overall parameters relevant for the fungal growth as percentage of surface overlay and number of conidia (Table 2). All the correlations are consistent; this supports the idea of the usage of image analysis as a tool for biomass monitoring in fungal mats on stone.

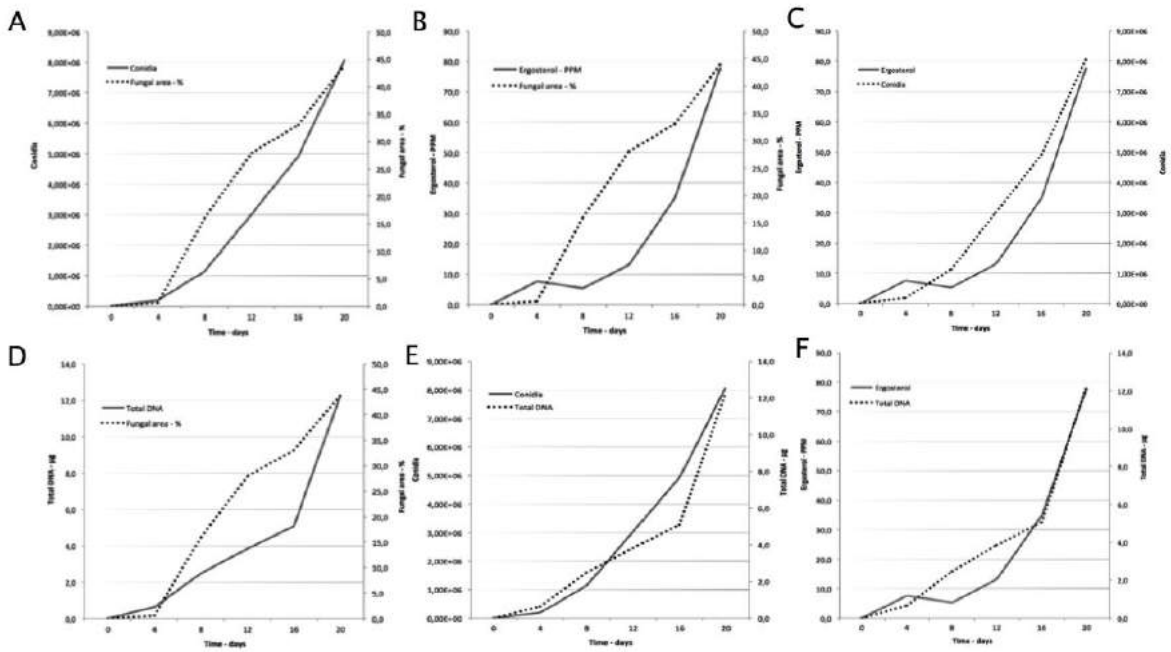

Fig. 6. All the six possible combinations of the measured indicators are shown in the figure above: A) conidia and fungal area; B) ergosterol and fungal area; C) ergosterol and conidia; D) DNA and fungal area; E) conidia and DNA; F) ergosterol and DNA.

Table 2. Correlation of ergosterol, DNA, conidia and percentace of overlay for the *A. tenuissima* fungal mats.

	Ergosterol (ppm)	DNA (µg)	Conidia	% overlay
Ergosterol (ppm)		0,001	0,002	0,035
DNA (µg)	0,975		0,001	0,012
Conidia	0,962	0,974		0,003
% overlay	0,844	0,909	0,953	

Epilithic and endolithic structure

Tuff is a very porous material, that allows a forward crypto-endolithic growth of mycelium. In order to investigate the architecture of the colony respect to the texture of the substratum, CLSM stacks were recorded after a calcofluor white 1% staining. Three points, in central medial and distal range of the surface were used to analyze the structure of the micelyum for all the experimental times. After the observation of the epilithic mycelium, the tuff tile was divided crosswise, and the inner section was also stained and observed in three points, corresponding with central medial and distal region. Cryptoendolithic growth was found in the cross section of the tile. To analyze the depth and the extension of cryptolithic hyphae the stacks were divided into ten "sub-stacks" of 128µm each. All data obtained by image analysis for the epilithic and cryptoendolithic growth of the hyphae in this experiment are reported in Table 3 and Table 4.

The analysis of horizontal distance reach and volume for cryptoendolithic hyphae is showed in (Fig. 6) for all experimental times. Horizontal distance reach of the crypto-endolithic hyphae is higher in the area of the inoculum and increases over the time. On the other hand, hyphal volume seems not to be related to the progress of fungal colonisation; in any case it appears to be greater in correspondance with the area of the inoculum. Taken together these findings suggest that penetration and development of mycelium substratum during the colonisation progresses inside the pores and the cracks into the substratum with a non-equal rate over the time, but shows a trend that is directionally spread from the inoculum to the periphery, and increases from the surface level to the inner layers.

Table 3. Analysis of various architectural parameters of the epilithic growth of *A. tenuissima.*

Time	4 days	8 days	12 days	16 days	20 days
Area average (µm²)	16440,838±5429,640	50837,799±14010,024	78704,779±20149,523	95792,747±38391,703	126038,136±101835,530
Perimeter average (µm)	9048,711±2950,369	26165,281±20347,945	37492,427±7082,183	31917,695±21141,928	34094,321±18069,552
Biomass average (µm³/µm²)	1,300±0,664	6,376±1,518	7,183±2,331	12,606±3,648	10,0391±9,537
Surface to biovolume ratio average (µm²/µm³)	1,263±0,770	1,045±0,600	0,846±0,120	0,567±0,155	1,380±0,357
Vol./MIP average (µm³/µm²)	27,903±16,067	52,716±9,978	37,264±5,431	57,379±13,061	29,538±6,806
Volume average (µm³)	466227,845±339083,658	2613410,089±622489,730	2943996,981±955534,406	5166759,051±1495326,980	4114519,363±3908517,337
Volume average (%)	0,798±0,683	3,751±1,176	3,644±1,167	7,731±2,635	6,984±7,403
Surface average (µm²)	543576,231±194105,270	2583113,214±1514983,192	2435541,741±572675,068	3048240,827±1584672,335	4752190,096±3859174,762
Thickness Biomass average (µm)	90,753±23,969	103,254±13,954	103,440±24,672	103,596±31,399	87,132±28,704
Max Thickness (µm)	240,580	215,360	247,000	198,458	296,120
Ra average	1,920±0,027	1,755±0,073	1,616±0,098	1,539±0,188	1,388±0,493

Table 4. Analysis of various architectural parameters of the crypto-endolitic colonization of *A. tenuissima.*

Time	Depth (µm) fom	to		Area tot. (µm²)	Area (%)	Vol./MIP (µm³/µm²)	Volume (µm³)	Biomass (µm³/µm²)	Surface to biovolume ratio (µm²/µm³)	Thickness (µm)	Ra	Surface
4 days	0	-128	427,620	10166,480	6,118	78,506	798134,273	4,985	1,531	30,084	1,872	1222407,010
	-128	-256	507,640	7885,545	4,746	78,109	615928,742	4,007	1,054	22,081	1,900	649599,050
	-256	-384	433,870	8085,655	4,866	66,058	534123,444	3,404	1,020	22,629	1,899	545057,130
	-384	-512	567,650	9684,964	5,829	75,249	728780,754	4,377	2,059	27,891	1,877	1500589,930
	-512	-640	445,210	5241,918	3,155	70,864	371464,312	2,324	2,065	10,480	1,936	767208,540
	-640	-768	300,290	6050,172	3,641	76,385	462143,055	2,830	0,971	10,569	1,924	449150,760
	-768	-896	176,340	2979,747	1,793	67,749	201876,033	1,286	0,946	5,040	1,962	191133,680
	-896	-1024	431,550	4364,876	2,627	73,180	319423,318	1,922	0,718	7,170	1,944	229372,390
	-1024	-1152	418,870	3705,144	2,230	59,872	221836,113	1,385	1,512	6,294	1,953	335459,210
	-1152	-1280	221,310	1493,001	0,899	74,812	111693,707	0,659	4,561	2,166	1,979	509441,170
8 days	0	-128	1902,410	13860,676	4,171	50,614	717325,170	2,300	3,994	12,593	1,911	2779095,240
	-128	-256	1944,310	12381,741	3,726	44,242	559336,453	1,724	1,579	10,132	1,923	1066342,080
	-256	-384	1805,520	10490,096	3,157	44,834	473629,468	1,524	2,073	7,552	1,932	853132,830
	-384	-512	1962,010	14262,459	4,292	40,522	631103,741	2,101	1,050	9,229	1,908	1017587,790
	-512	-640	2034,310	9930,410	2,988	43,371	450461,437	1,430	2,950	5,198	1,937	1353046,160
	-640	-768	1247,940	3922,451	1,180	22,461	172150,465	0,538	5,246	1,700	1,976	453494,280
	-768	-896	1214,160	4275,762	2,573	38,786	165837,650	1,035	2,539	5,666	1,946	421162,840
	-896	-1024	1050,290	1769,714	1,065	37,348	66095,173	0,412	4,527	2,127	1,977	299255,950
	-1024	-1152	380,140	1727,504	1,040	37,298	64432,799	0,402	1,871	2,656	1,978	120564,730
	-1152	-1280	8,750	128,194	0,077	30,367	3892,903	0,022	0,925	0,149	1,998	4210,146
12 days	0	-128	1702,200	7987,164	2,403	21,156	397795,578	2,553	2,110	10,883	1,845	867726,550
	-128	-256	1546,810	5382,621	1,620	20,386	391244,215	3,288	1,918	16,995	1,819	667172,180
	-256	-384	1934,320	6400,363	1,926	19,848	496156,728	4,411	2,183	16,491	1,791	1273145,140
	-384	-512	1762,920	5954,807	1,792	20,173	447758,448	3,878	1,121	15,849	1,816	702610,390
	-512	-640	2024,310	6714,596	2,020	54,588	613264,417	3,813	1,329	13,500	1,799	783362,430
	-640	-768	1747,340	6251,844	1,881	18,152	257682,021	2,095	1,303	7,373	1,855	405068,900
	-768	-896	1726,780	8149,754	2,452	19,544	296147,331	2,400	1,970	9,998	1,831	517061,650
	-896	-1024	812,030	3841,155	1,156	18,506	150633,803	1,371	3,574	5,451	1,894	468484,870
	-1024	-1152	411,460	2901,581	0,873	24,859	132362,284	1,027	7,807	6,276	1,911	324448,130
	-1152	-1280	21,670	948,955	0,571	42,024	39878,653	0,239	4,005	1,019	1,987	159729,600
16 days	0	-128	3283,030	17650,212	3,541	46,782	817006,551	1,698	1,507	8,740	1,925	1121194,029
	-128	-256	3506,190	63569,005	12,752	46,141	3234862,760	6,625	1,403	39,943	1,745	5159297,710
	-256	-384	3520,740	76338,470	15,314	53,224	4726247,329	10,354	1,487	47,609	1,673	5923035,290
	-384	-512	3704,470	80861,251	16,221	53,560	4251793,475	8,046	1,604	33,971	1,735	7828805,660
	-512	-640	3733,560	63251,654	12,688	49,913	3579946,291	7,642	1,223	40,291	1,740	5835929,700
	-640	-768	3535,970	41697,724	8,365	55,049	2328207,763	4,782	2,458	29,385	1,828	6062417,170
	-768	-896	2820,770	50590,054	10,149	60,155	2943178,814	6,080	1,873	34,325	1,792	4333216,260
	-896	-1024	2613,230	41685,215	8,362	54,134	2622432,155	5,422	3,523	24,625	1,827	5307693,240
	-1024	-1152	2420,810	26023,555	5,220	39,669	792944,595	1,639	2,576	9,336	1,892	1979543,800
	-1152	-1280	1224,930	12145,678	2,436	35,458	521584,224	1,058	1,261	4,012	1,950	669386,310
20 days	0	-128	3366,610	3952,930	0,793	357,873	1264876,745	8,843	3,669	70,941	1,617	4022523,380
	-128	-256	3503,530	4613,055	0,925	306,467	1324386,765	7,736	3,806	62,719	1,642	4819080,820
	-256	-384	3424,903	6663,388	1,337	196,166	1287471,452	8,999	3,528	68,938	1,593	4292355,170
	-384	-512	3600,620	5039,459	1,011	194,453	1098116,942	7,661	3,902	49,569	1,660	4320699,330
	-512	-640	3276,020	4992,558	1,002	164,475	923212,476	6,438	8,145	45,410	1,693	5954753,600
	-640	-768	2602,510	8026,630	1,610	69,995	633017,594	4,411	4,766	30,097	1,782	2817806,010
	-768	-896	2819,820	4155,774	0,834	100,868	430874,491	3,013	6,596	19,039	1,844	2305547,720
	-896	-1024	1827,250	3368,627	0,676	44,993	181814,473	1,265	9,450	7,287	1,927	1419368,520
	-1024	-1152	360,340	1130,302	0,227	21,021	28148,116	0,203	9,213	1,152	1,983	103175,600
	-1152	-1280	-	-	-	-	-	-	-	-	-	-

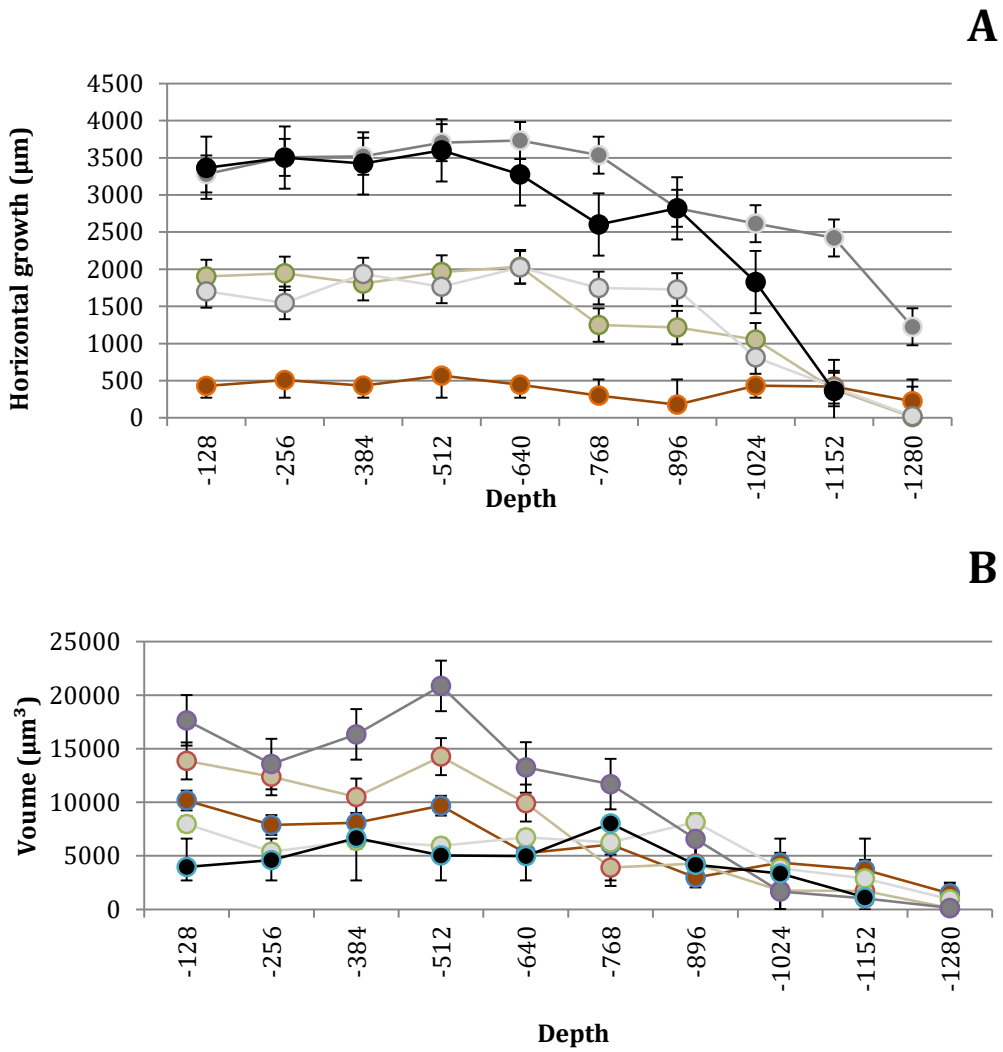

Fig. 6 Crypto-endolithic growth of *A. tenuissima* on tuff tiles. Horizontal growth **(A)** and volume **(B)** of crypto-endolithic hyphae; on *x* axys is reported sub-stacks separation, scale 128µm. Dark brown ●: day 4; light brown ●: day 8; light grey ●: day 12; dark grey ●: day 16; ● black: day 20.

Discussion

Colonization is one of the first steps leading to the subsequent formation of a biofilm on a material, which may result in the worst case in the destruction of the substratum. The development of microbial communities on stone surfaces tends to adequate to substrate topography and to fill depressions, fissures, and inter-granular spaces [26].

In our in vitro colonization experiments, the fungus *Alternaria* and *Fusarium* [27] show a loose net reticulum colonization on tuff stone. Hyphal branching increases over the time in the inner region of tile surface, that is associated with the inoculum; however the dispersal of conidia after the first week of observation deals to a remodeling of aerial mycelium structure. In in vitro experiments there is no external

perturbation, although in open systems fluctuating conditions and atmospheric weather can leave serious marks on the development of subaerial biofilms. It is probably in a scenario of conidia dispersal that the nest of hyphae already established on tuff stone can host other microrganisms, especially phototrophic communities. Roeselers and coauthors noted that the establishment of heterotrophic communities on rocks is possible even without the pioneering participation of phototropic organisms and may also facilitate the subsequent growth of photosynthetic populations [28]. The understanding of fungal establishment on stone rocks may also be considered as a fast biodeteriorating system both for the destructive penetrating power of fungi and for the ability to host heterogeneous communities over the time.

It is renewed that colonization may show trends that are associated with physical properties of materials such as roughness and porosity [9]. Neapolitan Yellow Tuff stone is an heterogeneous material, consisting of feldspar, mica, hydrated iron oxides and volcanic glass and with a incredibly high roughness and porosity [29]. These features determine a patchy substratum with a high water absorption coefficient, that is implied in moisture retainment. For this reason epilithic growth of *A. tenuissima* has also been monitored with CLS-Microscopy over the whole incubation time. Epilithic growth of the fungus is consistent with the overall growth biomass indicators relating to number of conidia, DNA and ergosterol, beside the surface overlay area. Image segmentation analysis provided a useful tool for an easy and fast determination of fungal overlay on stone surface, and the correlation with other biomass indicators may hopefully lead to the use of image analysis also for an in field application for the monitoring of natural biofilms.

Finally, the presence of feldspars and clays in a material provides points of weakness and significantly increase susceptibility to attack [30]. Few attempts have been reported for the measurement of endolithic growth in stone substrata [31]. For this reason crypto-endolithic growth of *A. tenuissima* has been measured with CLS-Microscopy by transversely splitting the stone tile in the middle and staining the inner section with a fluorescent dye for fungal walls. Our findings suggest that penetration and development of mycelium substratum during the colonisation proceeds inside the pores into the substratum with a non-equal rate over the time and that the spreading from the inoculum to the periphery increases from the surface level to the inner layers. This finding is corroborated by the fact that the apex of the hyphae is oriented in the sense of depth and not toward the surface, confirming that what we measured is

effectively a cryptoendolithic growth and not an hyphal growth that starts in a depression of the stone.

Conclusion

The study of fungal colonization on stone is an intriguing topic for biofilm research and conservation science. Heterotrophic communities may act as pioneers on bare rocks, and their presence can lead to the establishment of flourishing heterogeneous biofilms. Also, molds are renowned to be dangerous biodeteriogens, whose effects contribute massively to the fragmentation of the substratum.

In the present study a biological and quantitative survey on in vitro colonization of Neapolitan yellow tuff by *Alternaria tenuissima* was performed, thereby confirming the use of in vitro experiments for the understanding of biofilm establishment on stone.

Our tests have shown that *A. tenuissima* is able to colonize tuff tiles also in limiting conditions of carbon supply and that the yellow Neapolitan tuff is a very bioreceptive material, as previously assessed for the fungus *F. solani*. A combination of microscopy techniques (optical, epifluorescence, metallurgical and CLSM) and molecular techniques (DNA extraction, HPLC) has been successfully applied to assess the early steps of fungal colonization on stone substrata, especially to evaluate thickness and structural texture of the aerial mycelium. On the other hand, surface overlay of fungal colony and fine parameters of hyphal architecture were measured with computer image analysis, allowing also to analyze cryptoendolithic displacement and development of the hyphae into the stone substratum.

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CHAPTER 8

Colonization patterns on different lithotypes of an Oculatella subterranea *(Cyanobacteria) strain isolated from Pompeii archaeological site (Italy)*

Colonization patterns on different lithotypes of an *Oculatella subterranea* (Cyanobacteria) strain isolated from Pompeii archaeological site (Italy)

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Abstract The Cyanobcterium *Oculatella subterranea* inhabits hypogea and stone caves and is a pioneer of different stone substrata. In this study a strain isolated from the House of Marco Castricio (Archaeological park of Pompeii, Italy) was identified with a polyphasic approach and used for an in vitro colonization test. Fine architecture of *O. subterranean* biofilms was revealed as well as filaments orientation toward light source. This aim has been succeeded through CLSM microscopy and computer image analysis. Moreover, bioreceptivity of five different lithotypes, commonly retrieved in archaeological sites of Campania, was assessed for *O. subterranea*. Our results also indicates that the three-dimensional structure of *O. subterranea* biofilm is poorly affected by physical and geochemical features of substrates: in fact, the porous architecture of its biofilm was preserved, independently of the materials. It is opinion of the authors that a detailed knowledge of the three dimensional arrangement during the early steps of colonization can lead to the development of strategies specifically targeted to control the proliferation of this organism, improving the safeguard of cultural heritage.

Keywords bioreceptivity, *Oculatella subterranea*, lithotypes, image analysis, CSLM confocal microscopy, architecture of biofilms

Introduction

Biofilm adhesion is a manifold process regulated by the geo-chemical features of the material and the biological characters of the colonizer organism(s). Guillitte (1994) introduced the definition of bioreceptivity as "*the aptitude of a material (or any other inanimate object) to be colonized by one or several groups of living organisms without necessarily undergoing any biodeterioration*" meaning that there is an ecological relationship between the material and the colonizing organisms.

In a survey carried out at the archeological site of Pompeii (Italy) we have observed evident traces of bioweathering both on outdoor and indoor lithotypes of numerous

Roman Houses. A preliminary sample campaign has revealed that in many cases the prevailing organism occurring in biofilm was a filamentous, not branching Cyanobacterium, microscopically identified as *Oculatella subterranea*. This species was erected by Zammit et al. (2008), and has been reported as the typical inhabitant of caves and hypogeal (Hithsch et al. 2013). However, in Pompeii we found this organism under different light conditions, ranging from a dim light of internal rooms, to shadow spots lying on the outside walls of various buildings, and on different types of substrates (frescoes, tuff walls, Roman concrete, mosaic *tesserae*). Preliminary laboratory tests have shown that this strain grown on solid culture medium forming an open network of filaments with a high porosity. We have recently reported that the first steps of a surface colonization by phototrophic microorganisms can proceed according to two different patterns, defined compact or porous (Marasco et al. 2016); the first one presents an homogeneous structure, with a reduced number of empty spaces between cells or filaments, whereas in the latter, the spatial disposition of filaments allows the presence of numerous empty volumes. The aim of the present study is to verify the influence of the substrate on the biofilm architecture. On one strain isolated from the House of Marco Castricio and morphologically identified as *O. subterranea* we have carried out a molecular and phylogenetic analysis to confirm its full identity with this species. Then, we have performed a series of laboratory tests with the aim of assessing if the different features of the most frequent lithotypes occurring in Pompeii houses influences the first step of colonization by this strain. Five different substrata, namely: tuff, porphyry, brick, limestone and glass paste have been selected, and the tests were carried out following the procedure described by Del Mondo et al. (in press). With the aid of CLSM confocal microscopy we have tried to verify if the three-dimensional structure of *O. subterranea* could be influenced by the kind of lithotype. This point could shed light on the role of this organism as a pioneer in the establishment of biofilms on lithic substrates, since the structure and species composition of a biofilm depend on the spatial texture of the organism that starts the colonization (Curtis, Sloan 2004).

Material and methods

Petrographic data, Roughness and Porosity analysis

In vitro experiments were performed on 5 different lithotypes. The samples of Neapolitan Yellow Tuff were taken from the quarry of Quarto (Langella et al. 2000). The clay used for the production of brick samples comes from Sant'Agnello (NA) (De Bonis et al. 2013), the local manufacturing transformed the clay in little bricks, that were cut to obtain our samples. Porphyry comes from the quarries of Trentino Alto Adige (Martelli 1930; Pivko 2003). Vitreous samples were produced by ArteMarcia (http://www.artemarcia.com/). Limestone blocks are described in Marasco et al. (2016). All the lithotypes were cut in small blocks. Choosing the average size of the samples (20 x 20 x 10 mm) was based on the following elements: surface receiving the inoculum at the beginning of the tests was 2 cm^2; a small thickness (1 cm) was chosen to allow total and rapid humidification of the slab surface. The roughness parameters were evaluated on each block with an ALPA© RT-20 palmar rugosimeter, as described in the standard ISO 4287:1984. The measurements consisted of analyzing the surface of each block with 1600 sampling points (with a cut-off of 0.625 μm). All measurements were performed in triplicate, and data acquisition was conducted using the Measurement Studio Lite 1.0.3.96 software. To assess the density and the water absorption coefficient, the blocks were previously held at a constant value of temperature (21°±1 C) and relative humidity (51-55%) for two weeks. The average density (mean ± sd) of the different lithotypes was obtained by calculating the ratio weight/volume. The water absorption coefficient (WAC) (mean ± sd) was calculated according to Barberousse et al. (2007). According to Giesche (2006) the pore size and percentage of the 5 lithotypes were assessed through the technique of the mercury porosimetry (Autopore 4, Micromeritics).

Origin and identification of the strain

The experiments were conducted with the strain *O. subterranea* (ACUF 823; Fig. 1C) of the algal collection of the Department of Biology, University Federico II of Naples (www. acuf.net). Two apparently identical strains were isolated from samples collected on a mural painting of the Marco Castricio House, Pompeii Archeological site, Naples, Italy (Fig. 1A, B). DNA was extracted from liquid cultures of two strains with a modified Doyle and Doyle (1990) DNA extraction protocol and used for a

Polymerase Chain Reaction with primers targeting 16S rDNA (16S_long_F 5'-AGGATGCAAGCGTTATCCG-3'; 16S_long_R 5'-GGGGCATGCTGACTTGACG-3'). PCR was carried out in a 25 µl aliquot containing approximately 100ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x buffer), supplemented to give a final concentration of 2.5 mM MgCl2, 1.25U of Taq polymerase (EconoTaq, Lucigen), and 0.5 pmol of each primer. Amplification was run in a Applied Biosystem 2720 thermal cycler. The profile used was 5 min at 95°C, 33 cycles of 95°C for 30s, 60°C for 45s, and 72°C 45s, and a final elongation step of 7 min at 72°C. The amplification product (672bp) was evaluated on 1.2% (w/v) agarose gel in a electrophoretic run and purified using QIAquick® PCR Purification kit (Qiagen Inc, Valencia, CA, USA). Sequence reaction was obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), purified in automation using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA 01915 - USA) and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA). Product was analyzed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The amplification primers were used as the sequencing primers. Nucleotide sequence similarity was determined by using BLAST version 2.0 (National Center for Biotechnology Information databases).

A total of 41 sequences belonging to different *Oculatella* species and including *Leptolyngbya* as an outgroup were downloaded by Genbank nucleotide database; 16S rDNA multiple alignment was obtained by ClustalW (Larkin et al. 2007) with the addition of the two *Oculatella* strains isolated from Pompeii. The alignment, consisting of 611 sites, was then trimmed and adjusted by eye. Bayesian inference was obtained with MrBayes 3.2.0, running 2 millions generations and a sample frequency of 100 and using the General time reversible model (Tavaré 1986) with an invariable four gamma-distributed substitution rate categories to correct for among site rate variation (GTR+G+I); the analysis was stopped at an average standard deviation of split frequencies of 0.004086. The first 25% of sampled trees were discarded as burn-in before calculating posterior probabilities. The runs were evaluated with Tracer v1.6.0 and the final tree visualized and edited with FigTree v1.4.2.

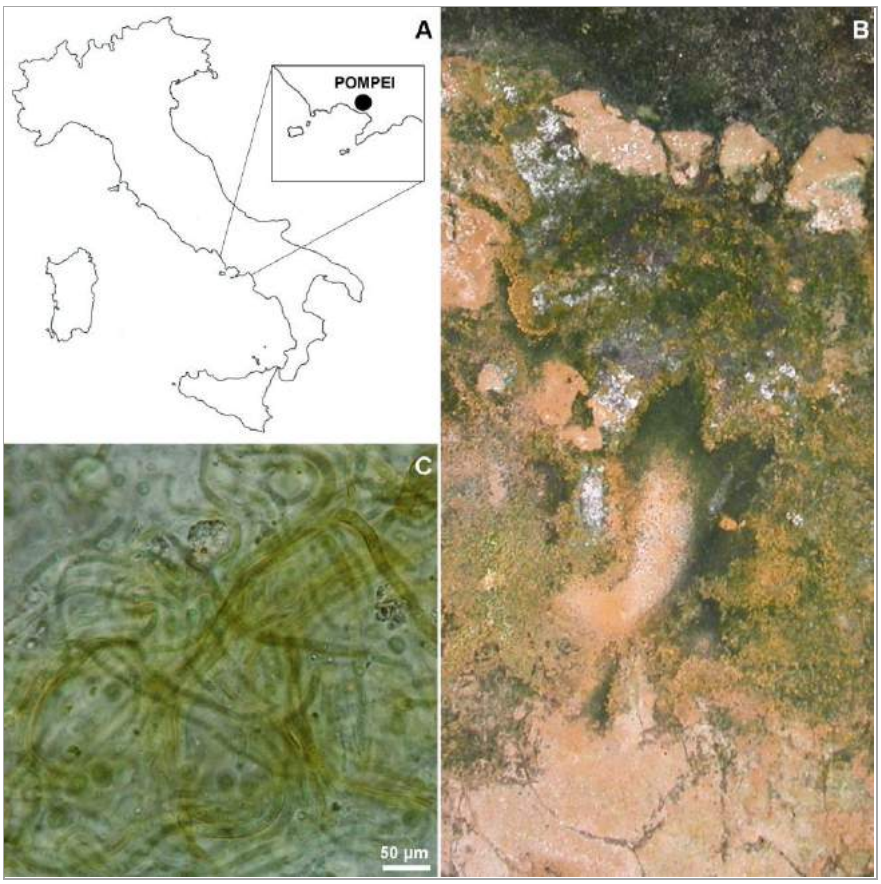

Fig. 1 Location of the archaeological site of Pompeii (A), detail of the some biofilms on the fresco of a wall (Marco Castricio House), macroscopic details (B) and microscopic view (C)

Accelerated colonization

A 20µl aliquot of a liquid culture of a *O. subterranea* in exponential growth phase (equivalent to 0,075 mg dry weight biomass), was inoculated with a sterile pipette on the middle of the upper surface of previously autoclaved blocks of tuff, porphyry, brick, limestone and glass paste.

A total of 25 blocks were inoculated for the primary bioreceptivity study. Five blocks of each material were observed weekly and growth rate of *O. subterranea* was followed by image analysis; the experiments lasted 12 weeks.

The experiments were conducted in sealed glass chambers filled for one third of their volume with sterilized distilled water to produce an environment with a relative air humidity close to 100%. A perforated ceramic grille was placed in each chamber at about 5 cm from the level of the water, and the inoculated blocks were placed on these grilles. The blocks were put in a climatic chamber at 20±2 °C, laterally

illuminated by growth light Osram white fluorescent lights placed sideways, with a 16:8 h light dark cycle, at an irradiance of 60 μmol photons /m^2/s. The irradiance was measured with a LI-COR Biosciences radiation (data logger LI-1400, quantum sensor LI-190).

Image analysis

Digital image analysis was applied to quantify the microbial growth on the blocks after the incubation time. The samples were taken out of the cell chamber and placed on filter paper to remove excess water. The photographic recording of each lithotype block was performed in triplicate with a digital camera (Nikon D5100 with Nikkor 50mm objective). The conventional RGB color images obtained with the digital camera were used to quantify the colonization area on each lithic sample. The program *Trainable Weka Segmentation* (Arganda-Carreras et al. 2017; Vyas et al. 2016), a plugin of open source image processing package *Fiji* (Schindelin et al. 2012; and also http://www.fiji.sc), was used to evaluate the colonized areas of each photograph were measured. Then, the empty and colonized areas on each *tessera* were identified by means of the shape and color, respectively.

CLSM Observations

At the end of the experiments (12^{th} week) each block was also observed with a Confocal Laser Scanner Microscope (CLSM) Zeiss LSM 700 (software Zen 2011) by capturing images at 10x in order to distinguish the three dimensional structure of the microbial biofilms. The autofluorescence of phototrophs and EPS were recorded in the two channels simultaneously; red channel for pigment autofluorescence (chlorophyll *a* and phycobilins), with an excitation beams at 543 and 633 nm and emission at 590-800 nm (red channel). The acid polysaccharides of the extrapolymeric matrix (EPS) were detected using the concavalina-A with the Alexa 488, at an excitation beams at 488 nm and emission at 553-636 nm (green channel). The images from stacks were captured at 5-6 μm intervals. The substratum area of the image stack was 1024 x 1024 pixels. The number of images in each stack varied according to the thickness of the biofilm.

The open source image processing package Fiji (http://www.fiji.sc) was used to evaluate the area of all stacked CLSM images, and to obtain 2D Maximum Intensity Proiections (MIPs). The images have been previously converted to 8-bit and then

resampled by using the tool *Threshold* (Baveye 2002; Lepanto et al. 2014; Kuehn et al. 1998). The stacked images were then analyzed with *3D Manager* (Ollion et al. 2013) and *Comstat2* (Heydorn et al. 2000) for the characteristic three-dimensional biofilm. Biofilm samples that showed better growth were subjected to further image analysis to characterize algal filament networks. To estimate the local orientation (Liu 1991) of the algae filaments, we used the ImageJ plugin *OrientationJ* created by Daniel Sage (http://bigwww.epfl.ch/demo/orientation/), following their respective instructions. To obtain the distribution of fiber diameters we used the plugin *DiameterJ* (Hotaling et al. 2015). In addition, the plugin Fractal Dimension, applying a box-counting algorithm, evaluated the *fractal dimensions* of each organism pattern (www.bonej.org/fractal).

Results

Petrographic data, Roughness and Porosity analysis

The lithological characteristics of brick, limestone, glass and Neapolitan yellow tuff used in this work have already been described in Langella et al. 2000; Marasco et al. 2016; Verità 2014; De Bonis et al. 2013, respectively. The chemical composition of porphyry consisted of oxides of aluminum, titanium, iron, calcium, magnesium, potassium, sodium and most of silicon oxides (Martelli 1930; Camera di Commercio IAA di Trento 2008). The mineralogical components of glass blocks were: quartz, sanidino, plagioclase, subordinately biotite, redox, and pasta glass (Stern 2008; Campanella et al. 2007). For each lithotype, mean density, water absorption coefficient (WAC), roughness and porosity are reported in Table 1.

Table 1 Density, water absorption, roughness and porosity of different materials on which the microbial strains grew

Lithic sample	Mean density (g cm^{-3})	Water absorption coefficient (WAC) g dm^{-2} $min^{-1/2}$	Ra	Rq	Rz	Rt	Porosity (%)
Brick	2.04	29.81^{-5}	2.44	3.33	16.70	27.6	51.80
Porphyry	2.55	3.55^{-5}	6.69	8.2	33.47	51.95	45.90
Tufa	1.46	49.28^{-5}	17.63	21.4	77.99	97.56	56.63
Glass	3.41	1.17^{-5}	1.13	1.35	5.19	7.05	48.23
Limestone	2.61	2.13^{-5}	2.97	3.70	14.14	23.89	33.39

The average density of lithotypes used in this experiment is increasing according to the order: tuff, brick, porphyry, limestone and glass. The same order of materials, but

an opposite ranking was also found as far as concerns WAC. The characteristics of the surfaces of the different lithotypes can be derived from porosity and roughness (Table 1); Figure 1 shows the cumulative intrusion (mL/g) of mercury relative to the pores diameter (nm) of the different materials. Both indicators concurr to indicate a high irregularity of yellow tuff, followed by brick. Glass is the material with the lowest pore sizes (Fig. 2) and surface roughness (Table 1).

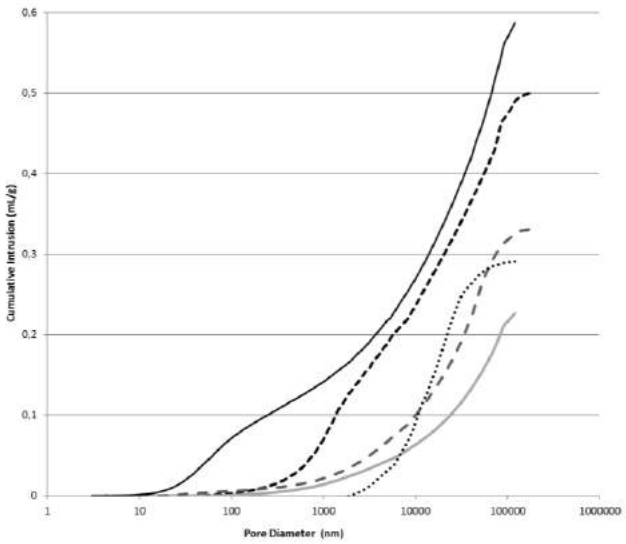

Fig. 2 Cumulative intrusion of Hg in relation to the pore size of the selected five substrata. brick (- - -), porphyry (– – –), glass (· · ·), tuff (── black line), limestone (── gray line).

Strain identification

Molecular analysis attributed the two strains isolated form a fresco of the House of Marco Castricio (archeological site of Pompeii, Italy) to the species *O. subterranea*, with a 99% similarity score on BLAST (https://blast.ncbi.nlm.nih.gov). Bayesian inference also confirmed the relationships among the extant *Oculatella* species, providing a robust clade for the species *O. subterranea* that cluster with a high posterior probability support (Fig. 3).

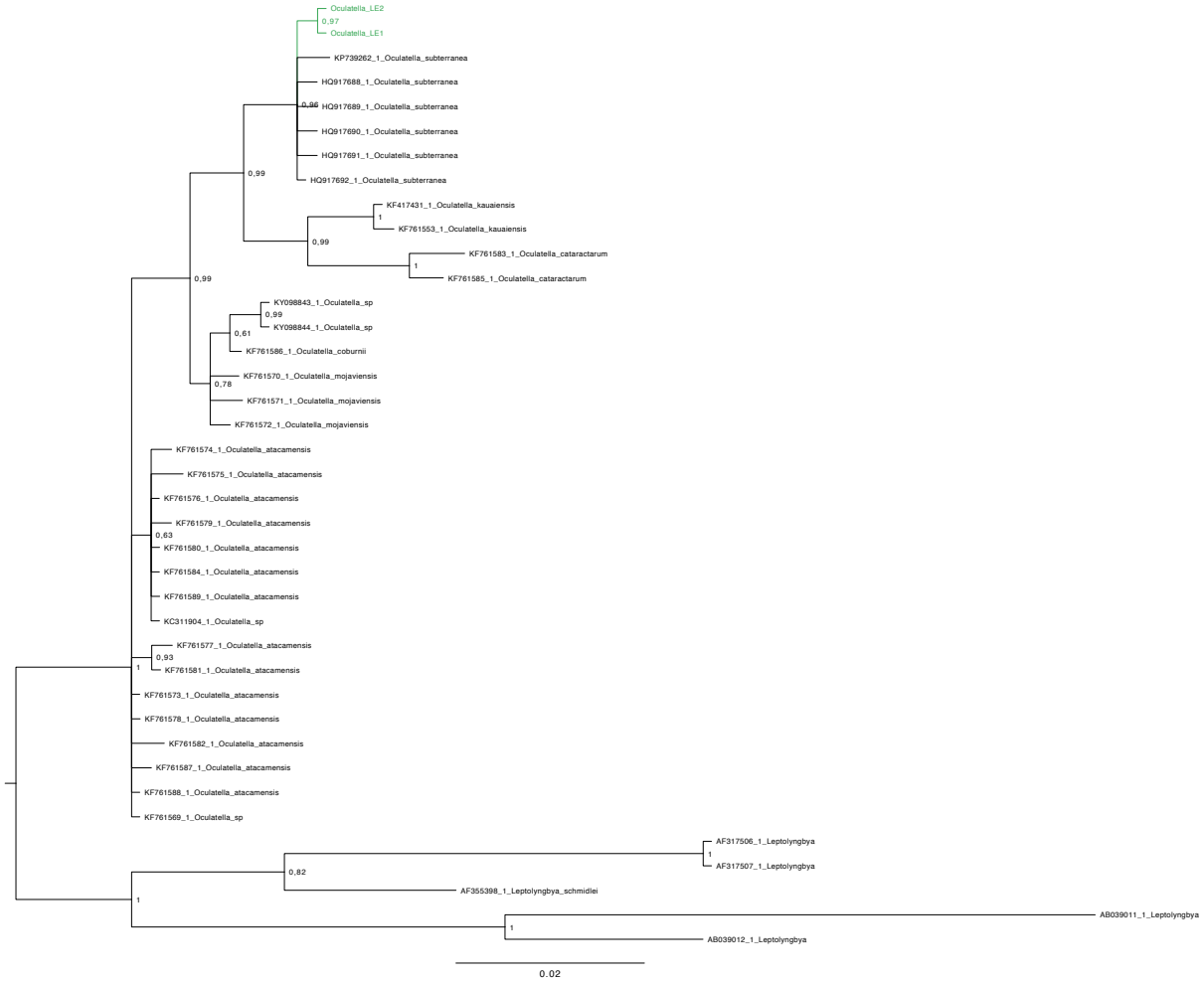

Fig. 3 Phylogenetic tree of *Oculatella* species. The identities of strains correspond to those given in the databases and accession numbers are given in parentheses after the taxonomic assignment. The phylogram was obtained from partial sequences of 16S rDNA. Posterior probabilities from Bayesian analysis by numbers on branches

Accelerated colonization

O. subterranea grew on all substrates, but the rate of colonization depended on the physical features of the block surfaces. In our the experimental conditions, growth was no more recorded on glass and limestone from 7th week to the end of the experiment (Fig. 5); on the contrary, larger biofilms consisting of many layers were found on brick, yellow tuff, and, to a minor extent, on porphyry. Brick was the material on which the growth of *O. subterranea* was faster: in the second week the surface coverage was already 48.18%, and at the end of the experiment the biofilm had reached 100% (Fig. 4, 5). The growth of the cyanobacterium on the yellow tuff also produced a final surface coverage of 100%, although with a slower rate of colonization. The growth of *O. subterranea* on porphyry showed intermediate

characters between brick and tuff on one side, and limestone and glass on the other, with a final coverage of 36.27% of the lithic surface (Fig. 5).

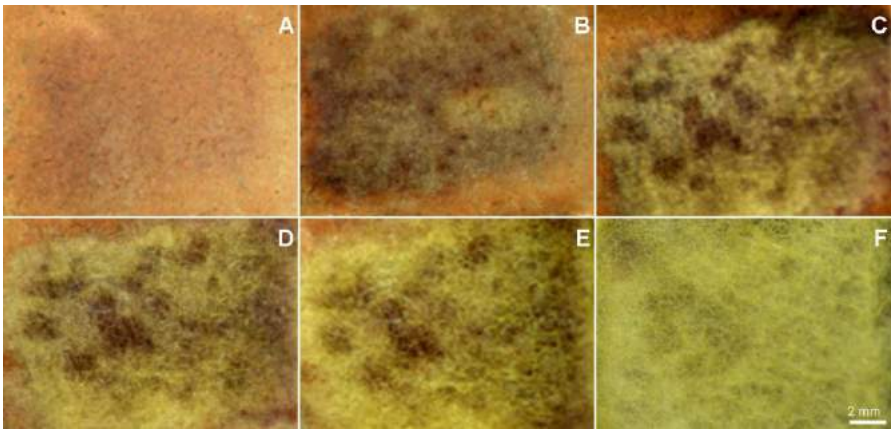

Fig. 4 Example of colonization on a brick block by *O. subterranean*; each photograph corresponds to a temporal progression (1 week) of biofilm for the first five weeks (A-E); in F the surface overlay at end of the experiment (12th week)

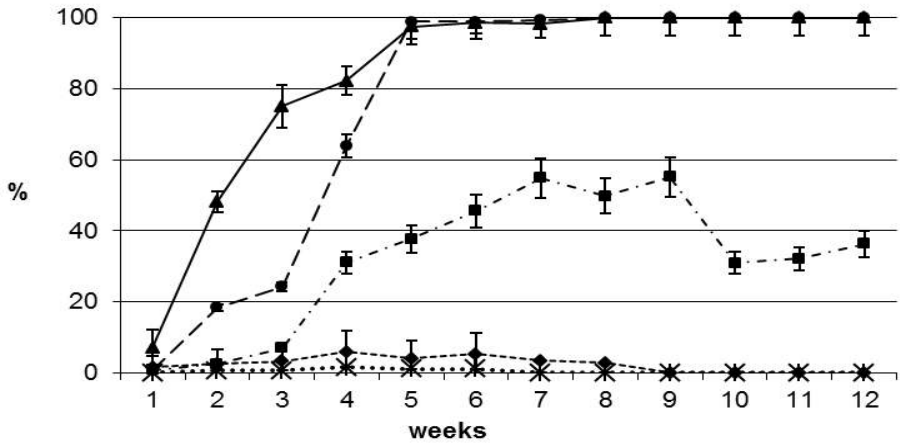

Fig. 5 Evolution of surface colonization (%) of *O. subterranea* on the five different substrata. Brick (——), glass (• • •), limestone (- - -), porphyry (· - · -), tufa (— — —)

In order to obtain a quantitative estimate of biofilm structure, bio-volume, maximum thickness, substratum coverage and roughness parameters were extracted from the confocal stack images (Table 2). Despite the differences in geolithological properties of the six chosen materials, in all of them *O. subterranea* filaments generated a reticulated mat, whose biomass, thickness, air-exposed roughness surface and other structural elements exhibited different values.

Table 2 Analysis of various architectural parameters of *O. subterranea* biofilm

Lithotype	**Brick**	**Glass**	**Limestone**	**Porphyry**	**Tuff**
Substratum coverage photo (%)	100 ± 0.25	0	0	36.27 ± 2.43	100 ± 0.54
Substratum coverage MIP (%)	100 ± 1.16	0.81 ± 0.3	0.88 ± 0.51	30.82 ± 5.62	100 ± 1.94
Biomass ($\mu m^3/\mu m^2$)	30.17 ± 3.23	0.49 ± 0.22	0.58 ± 0.31	3.673 ± 0.92	12.95 ± 3.52
Roughness coefficient (Ra)	28.78 ± 2.71	1.72 ± 0.84	2.45 ± 0.53	31.74 ± 2.18	17.42 ± 5.82
Mean thickness (µm)	21.18 ± 1.27	2.27 ± 0.28	1.67 ± 0.41	2.78 ± 0.74	16.27 ± 2.65
Max thickness (µm)	35.94	3.83	2.72	6.58	46.27

As expected, the *O. subterranea* biomass on brick and tuff was larger and thicker than those on the other materials; moreover the roughness of the biofilm surface on the above mentioned materials showed high values, and produced a visual patchy appearance, dictated from the arrangement of *O. subterranea* filaments on the surface of substrates (Fig. 6).

If we take into consideration the three materials that showed a higher bioreceptivity to the cyanobacterium, the vertical profile of *O. subterranea* mats starting from the surface of the blocks showed an area/perimeter ratio that increased with increasing height, then decreasing in the top layers (Fig. 7). This trend was found on brick, yellow tuff and porphyry, even though the values of the area/perimeter ratio found in biofilm growing on brick exceeded by at least one order of magnitude those typically measured in yellow tuff, and porphyry.

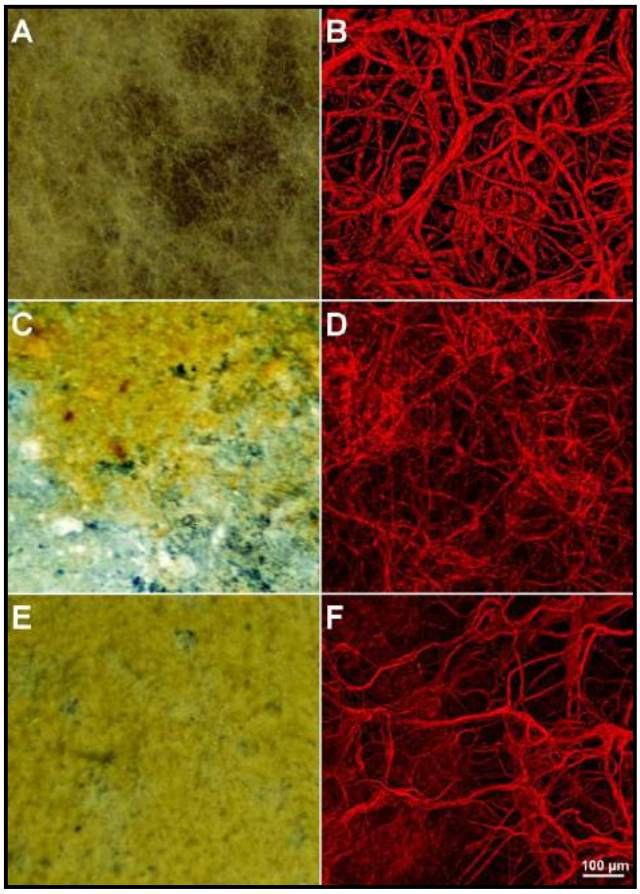

Fig. 6 Superficial growth of the *O. subterranea* biofilm on brick (A), porphyry (C) and tuff (E); the 3D reconstruction of the z-stacks (B,D,F) with one channel (autofluorescence red)

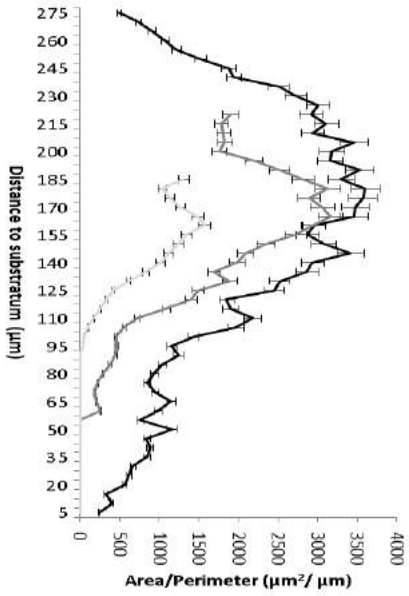

Fig. 7 Vertical profiles of the area/perimeter ratio of *O. subterranean* biofilm grown on: brick (black ——), porphyry (light grey ——), tuff (dark grey ——)

The net-like structure of each biofilm was analyzed through the determination of filament orientation, diameter, mean length, number of intersections, number and dimensions of voids, determined with different plug-ins on all the available Z-stacks (Table 3). We assumed that algal filaments were mainly located in the circumferential–longitudinal plane, which allowed us to flatten the 3D data to 2D (Hotaling et al. 2015). *O. subterranea* grows on the blocks by building a porous structure made by single filaments intermingled with bundled filaments, that contour around void spaces, whose number and maximum area progressively increases from brick, to yellow tuff and porphyry. Filament length and intersection data concur to indicate that on brick the spatial arrangement of *O. subterranea* is more regular, with a prevalence of shorter filaments that can form large bundles, with a parallel course and a relatively low rate of overlapping, whereas yellow tuff and porphyry produce a more reticulated pattern of surface colonization. Finally, as far as concerns spatial orientation, the predominantly axial orientation of *O. subterranea* filaments on the blocks fluctuates from -11.46° to +18.84° (Fig. 6). The network of filaments produced on porphyry did not show a preferred horizontal orientation, in contrast to the biofilm grown on brick and yellow tuff, that had a more pronounced character of orientation (brick: 10.41°; yellow tuff: 18.84°).

Table 6 Analysis of various parameters of filament network of *O. subterranea* biofilm

	Brick	**Porphyry**	**Tufa**
Fiber length (µm)	30898.18	42745.94	59075.00
Mean pore area (µm^2)	284.69 ± 101.53	150.62 ± 41.43	451.01 ± 212.76
Min. pore area (µm^2)	33.05	18.26	36.98
Max. pore area (µm^2)	410.05	4558.07	13036.24
Number of pores	512	787	673
n° of intersection	3967	7171	7182
Intersection density (ints / µm^2)	0.004	0.015	0.007
Char. length (µm) (lungh tot fibr / n° fibr sovrapp)	2.967	9.92	15.29

Discussion

The susceptibility of stone and mineral-based material to bioweathering can be influenced by chemical and mineralogical composition, physical form, and geological origin (Hutchens 2009; Turick and Berry 2016). Rougher textured surfaces also tend to collect more algae, presenting empty spaces to accommodate potential new organisms: Characklis et al. (1990) noted that the extent of microorganism colonization appeared to increase as the surface roughness increased. Recently, Vázquez-Nion et al. (2018) reported that open porosity and capillary water plays a pivotal role in the development of biofilms on lithic materials, whereas a minor contribution was provided by chemical properties of the stones. All these findings have been confirmed also in our experiments: *O. subterranea* formed larger and multilayered biofilm on substrates with high WAC, roughness and porosity, namely brick and yellow tuff. However, our results suggest also that the 3D structure of *O. subterranea* biofilm during the early stages of colonization is scarcely affected by physical and geochemical features of substrates: in fact, the porous architecture of *O. subterranea* biofilm was preserved, independently of the materials. On the other hand, an increasing heterogeneity of the reticulate mat was found among biofilms developed on brick, yellow tuff and porphyry, even though it is very difficult to establish when two biofilms are different. This distinction should be based on an arbitrary definition of a significant difference (Beyenala et al. 2004); however our data indicate that in larger biofilms growing on brick porosity decreases and cell bundles are larger. This pattern should reduce the average diffusion distance that a nutrient needs to travel to get filaments (Yang et al. 2000) ensuring that each cell can receive the required feeding.

The genus *Oculatella* is characterized by a reduced production of EPS (Zammit et al, 2012), and also in the course of our experiments we observed only traces of external polysaccharides, thus suggesting that the amount of EPS produced are more dependent on the intrinsic characteristics of the organisms rather than on the physical and chemical features of the substratum. The spatial organization of mature *O. subterranea* biofilm on different substrates is similar, a spindle-shaped distribution of filaments was observed on each surface, and is probably consistent with a response to light availability. Filaments initially grew horizontally, but after one week orient themselves normal to the surface of the mat, as also observed in the case of lab tests

on stromatolites spatial geometry (Petrof et al. 2010). This configuration probably permits a better exposition to light irradiance, whereas the numerous voids could act air trappers, allowing an effective carbon dioxide distribution along the filament layers.

Cyanobacteria are resistant to desiccation and high solar irradiation (Garcia-Pichel et al. 1993; Roy et al. 1997), and their growth on stone surfaces is very diffused in the tropical and sub-tropical countries (Gaylarde and Gaylarde 2005; Gaylarde et al. 2012). At Mediterranean latitudes *O. subterranea* is largely diffused in hypogean habitats, but our findings reveals that can grow also on different substrata under the daylight, although not directly exposed to sun irradiation. In these situations, *O. subterranea* can give a major contribution to the first colonization of different hard substrata, and its net-like structure may facilitate the following establishment of other microorganisms. Recently, new strategies of control as nanographene oxides (Bruno et al. 2014), and new LED illumination systems (Bruno and Valle, 2017) gave promising results in the control of the growth of several cyanobacteria, including *O. subterranea.* In our opinion, a detailed knowledge of the three dimensional arrangement during the first stages of colonization can lead to the development of strategies specifically targeted to control the proliferation of this organism, improving the safeguard of cultural heritage.

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CHAPTER 9
GENERAL CONCLUSIONS

The study of subaerial biofilms may open new insights for a wide range of applications. Cell-adhesion to the substratum in different environments, fast evaluation of biofilm composition in solid-matrix bioreactors, identification of urban pollution bio-indicators, design of new materials and antifouling coatings are all research field which can be implemented with a deeper knowledge of subaerial biofilms structure and ecology.

A further remarkable intent is the preservation of cultural heritage: it represents a unique and non-renewable resource to the societal and economic well-being of communities. Historical buildings, archaeological sites, stone monuments, wall paintings and frescoes are all subjected to the deterioration caused by living organisms, especially microbes; depending on several factors as light exposure, humidity and human intervention, a variety of eukaryotic and prokaryotic heterotrophs and autotrophs can thrive at rock-atmosphere interface.

It is well known that some microrganisms are pioneers in colonization of virgin substrates; however it is still not clear if their ability is related to specific metabolic and morphologic features or if their attachment and proliferation on substrates is pushed by favorable environmental conditions. A huge step in this direction has been provided by in vitro colonization experiments, which made possible to selectively study the ability of microrganisms to attach and colonize as well as the refractoriness of the surface subjected to colonization. Till recent times it was commonly accepted that Cyanobacteria were the only microrganisms able to successfully colonize stone surface, due to their poor metabolic exigencies and the ability to grow also in dim light. However, nowadays there is strong evidence that also heterotrophic eukaryotes can act as first colonizer, enhancing the formation of mixed consortia.

In our in vitro colonization experiments, the pioneer attitude of the fungi *Fusarium solani* and *Alternaria tenuissima* as well as the cyanobaterium *Oculatella subterranea*, was tested and monitored for a short-term period. Molds were isolated from the Villa of Poppea in the archaeological site of Oplontis while the cyanobacterium was isolated from the House of Marco Castricio in the archaeological

park of Pompeii. Through the use of many variants of microscopy included CLSM and computer image analysis it has been possible to depict fine structure and architecture of the studied microrganisms, in a controlled environment that reproduced realistically the conditions of the respective sampling points. Besides, additional investigations were performed on characteristic features of these microrganisms, as the crypto-endolithic growth of the fungus *A. tenuissima* or the reorientation of filaments toward light source of *O. subterranean*. These achievements can elucidate the treatment and the restoration strategies of weathered monuments, in which these organisms and others related are likely to occur.

In fact, beside the standard operation of samplings and identification accomplished for several archeological sites in Campania, also a deep documentation of case studies and reviews was collected, in order to list the organisms that occur on weathered monuments in European countries. From this list it has been found that there is no phylogenetic conditioning in the colonization of stones, id est many and different Phyla and Ordines of eukaryotic algae and fungi and prokaryotes are identified as living on stone substrata. This finding encouraged the construction of nucleotide alignments for a selection of barcoding markers, in order to design group-specific oligos, specifically aimed to the biofilm characterization.

New investigation tools for the investigation of the biofilms are indeed required, that can satisfy the needs of small amount of sampling material to be analyzed in non-invasive and highly reproducible assay. To achieve this purpose in the present work it has been discussed the employment of the designed group-specific primers to be used in qPCR reaction for the quantification of biofilm components, as well as group-specific fluorescent internal probes. Quantitative PCR is extremely sensitive and reproducible, and the proposed oligos were projected in a way that is fully compatible with a multiplex reaction assay to obtain relative levels of abundance in biofilms for each selected group of microrganisms. Moreover, probes design can be targeted also for specific genera or species, increasing the specificity of the assay to particular situations.

A further proposed tool is the characterization of microbial diversity through the use of flow cytometry. Although this technique has been widely used in ecology for the identification of marine communities, there are only shy attempts for its use with microbial mats of subaerial biofilms and aeroterrestrial algae. In the present work, biofilms sampled from the Stufe of Solfatara (Pozzuoli, Italy), a harsh volcanic

environment characterized by the presence of few species sharing common characters of tolerance to acids and high temperature. Phototrophic components were analyzed with flow cytometry, which allowed the sorting of the two Genera *Cyanidium* and *Galdieria,* unicellular Rhodophyta whose morphological identification is obtained by poor and overlapping diacritic characters. Confirmation of cytometry results and precise assessment of the species was later obtained with the use of novel-designed species-specific primers targeting plastidial gene rbcL, and here developed for the identification of Cyanidiales.

Taken together, the findings reported in the present work represent an encouraging advance in the study of subaerial biofilms with a number of techniques that rely on small amounts of samples and improve the sensitivity of characterization of biofilms. Moreover, the use of image analysis applied to the study of fine architecture of microrganisms has been coupled with the setting of reproducible in vitro colonization experiments, defining behavior and features of microscopical growth of microrganisms on stone substrates.

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AKNOWLEDGEMENTS

Firstly, I would like to express my most sincere gratitude to both Prof. Gabriele Pinto and Prof. Antonino Pollio for the continuous support of my Ph.D. study and related research: their guidance helped me in all the time of research and writing of this thesis. Moreover, their far-beyond-the-average human value has been only even with their incredible patience, motivation, and immense knowledge.

Besides my advisors, I would like to thank Dr. Antonino De Natale, for his insightful comments and encouragement, but also for incenting me to widen my research from various perspectives. My sincere thanks also go to Dr. Claudia Ciniglia for her inclusive and enthusiastic support and for her kind consideration.

I am also very grateful to Prof. Antonio Porcellini for enlightening me the first glance of research and for all the stimulating discussions and advice about science and molecular biology. His out-of-the-box perspective is something precious that I wish to gain as a researcher someday.

I thank all my fellow colleagues and students for all the fun we have had and for all the science we made in the last three years: you all made me feel like in a wonderful team, and I wish you all the best in career. It is hard to name here the over thirty labmates who shared the past three years in the lab with me, but for the much time we spent together and their friendly attachment I cannot hold myself back from mentioning Dr. Manuela Iovinella, Dr. Dora Carbone, Dr. Jairo Moreno, Mariano Santoro and Luigi D'Elia.

Also I thank all of my friends and colleagues in the Department of Biology: in particular, I wish to thank Dr. Candida Zuchegna and Dr. Antonella Romano for their supportive friendship and for helping in all the tricky situations.

Last but not the least, I would like to thank my family: my parents and my sister for supporting me spiritually throughout writing this thesis and my life in general, with unchanged love and understanding.