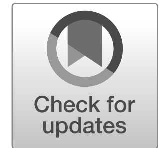
Annals of Microbiology (2019) 69:1097–1106
https://doi.org/10.1007/s13213-019-01480-9

CORRECTION



Correction to: Novel qPCR probe systems for the characterization of subaerial biofilms on stone monuments

Angelo del Mondo[1] · Antonino de Natale[1] · Gabriele Pinto[1] · Antonino Pollio[1]

Published online: 28 May 2019
© Università degli studi di Milano 2019

Abstract

Purpose A deep survey of biodeteriogen microorganisms reported on stone monuments in Europe has been performed based on the available literature dating back to over 30 years. The aim of the present study is to obtain accurate oligos for the characterization of subaerial biofilms on the basis of the most comprehensive collection of reports and case studies regarding subaerial biofilms, with particular regard to phototrophic and non-phototrophic bacteria, eukaryotic algae and molds.

Methods The obtained lists for eukaryotic algae, phototrophic and non-phototrophic bacteria and 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 bibliometric diversity, multiple nucleotide alignments were produced and primers were designed for a qPCR assay.

Result Primers were designed on conserved regions flanking a variable region, specific for each of the studied groups of microorganisms. Standard curve for absolute quantification relative to each group were determined for four markers. Then, variable regions in the alignments were used to design fluorescent internal probes for qPCR aimed for a multiplex reaction in which relative abundance can be determined.

Conclusion The authors propose this kind of cost-effective approach in the study of biofilms for the estimation of algae, molds and bacteria both for direct in situ analysis and in vitro simulation.

Keywords DNA-PCR analysis · Real time · Microbial mat · Quantitative determination · Monuments

Introduction

Subaerial biofilms on stone substrata

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 films are composed by densely packed microorganisms that live in self-organized structures of micron to millimeter scales. Made up of a multitude 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). Typically, 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 et al. 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

The online version of the original article which can be found at https://doi.org/10.1007/s13213-019-01461-y is not the updated version. To avoid any misinterpretation, the whole article is published below in full.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13213-019-01480-9) contains supplementary material, which is available to authorized users.

✉ Angelo del Mondo
angelo.delmondo@unina.it

[1] Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cintia, 80126 Naples, Italy

populations (Roeselers et al. 2008). 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).

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 (Scheerer et al. 2009). Tolker-Nielsen and Molin (2000) noted that every microbial biofilm community is unique although some structural attributes can generally be considered universal. Here, a novel approach for the characterization of subaerial biofilm is described by focusing on the universal components in epilithic communities.

The identification of the phototrophic and heterotrophic components in 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 structure, 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 in 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 identify molds (Op De Beeck et al. 2014). DNA extraction coupled with PCR reaction regarding barcoding genes allows the efficient identification of microorganisms. Aside the use of 18S marker for green algae identification, a number of plastidial markers have been proposed, rbcL and TufA above all (Hall et al. 2010; Saunders and Kucera 2010; Du et al. 2013). Similarly, for Cyanobacteria, the use of cpcA (C-phycocyanin alpha chain) has been proposed (Neilan et al. 1995; Miller and McMahon 2011) and dinitrogenase reductase nifH genes for barcoding (Zehr and McReynolds 1989; Poly et al. 2001). In addition, 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 present problems in pattern visualization on agarose gel and other major limitations (Neilsona 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. Profiling microbial mats through the quantification of microbial groups which drive biofilm formation could give novel insights in the study of microbial ecology and biodeterioration. Fluorescent internal probes are required to increase specificity and sensitivity of the assay, but they may also open a new frontier by obtaining relative quantification in multiplex assay. Different combinations of primers and probes may target for specific taxa or metabolic groups, offering new insights for biofilm characterization, such as monitoring of biodeterioration, determination of ecological successions in biofilms, and the assessment of biocide efficiency. The aim of the present study is to obtain accurate oligos for the characterization of principal components in subaerial biofilms thriving on stone. This has been done on the basis of a very inclusive collection of literature reports and case studies regarding subaerial biofilms collected on stone monuments, with particular attention to phototrophic and non-phototrophic bacteria, eukaryotic algae, and molds: the authors hereby propose the use of novel designed oligos for the characterization of most common microbial groups forming 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, for a time range going from 1967 to 2018. All taxa obtained by case-study literature were listed and grouped by Genera, accounting for 63 Genera of Bacteria, 57 Genera of Fungi, 77 Genera of Chlorophyta, 62 Genera of Cyanobacteria, and 29 Genera of Diatoms (Supplementary materials Tables S1, S2, S3, S4, and S5). Identifications of microorganisms carried out by several methods in the cited case-study literature papers were listed at the taxonomic level of Genus; for each monumental or environmental site in case of multiple species for the same Genus, only one has been considered for the counting, in order not to bias the extant data. Then after, Genera were summed up with the criterium 1 site: 1 Genus. Subsequently, higher taxonomic information

and morphological features were collected for each Genus and used to point out percentages. Taxa of uncertain attribution were excluded from the analysis. Subsequently, from three up to six sequences for each Genus were downloaded by GenBank database when available, in order to build multiple nucleotide alignments. This operation was re-iterated for each barcoding marker investigated in the present study. Since diatoms are greatly related to historical and monumental stone fountains but scarcely represented in subaerial biofilms in which water is less present, after their bibliometrical assessment were excluded from the marker selection. Similarly, Archaea and Red algae (Rhodophyta), which are scarcely described in subaerial biofilms and lesser described in literature, were also excluded by marker selection.

Multiple nucleotide alignment for the selected markers

Seven candidate molecular markers (Bacteria: cpcA, 16S, NifH; Green microalgae: rbcL, tufA, 18S; Fungi: ITS1) were chosen for the three selected groups of microorganisms. 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 multiple nucleotide 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. Primers were designed in order to obtain amplicons of maximum size of 200 bp. Primers have been located in regions of 100% nucleotide conservation which contained a sequence selectively variable for the organisms of interest, suitable for designing an internal fluorescent probe. In silico PCR simulation was performed with Amplify4 software v.0.9.5 (Engels 2015) that also provided the annealing temperature for each couple of primers. The oligos were synthesized by IDT Company.

DNA extraction and PCR

The DNAs were extracted by ACUF collection (www.acuf.net) strains with CTAB DNA extraction (Doyle and Doyle 1990). PCR were carried out in a 25–μl aliquots containing approximately 50 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10× buffer), supplemented to give a final concentration of 2.5 mM MgCl2, 1.25 U of Taq polymerase (EconoTaq, Lucigen), and 0.5 pmol of each primer. Amplifications were run in an Applied Biosystem 2720 thermal cycler. The profile used was 10 min at 95 °C, 15 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s with annealing increasing of + 0.5° at each cycle, followed by 20 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s and a final elongation step of 10 min 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 absolute DNA quantification

Due to the issues related to variability and sequence availability in database, only four markers were tested in this phase, namely 16S, rbcl, tufA, ITS (Table 1). Six dilution series of mixed DNAs at eight different concentrations have 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 (Supplementary S6). The PCR reactions were carried out using the RealAmpTM SYBR qPCR Master (GeneAll® Biotechnology), 1.5 pmol of each primer, and 1 μl 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 15 s, 60 °C

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′	23 bp
	Tufa_R	5′-TCATATTTATCTAAAGTTTC ACG-3′	20 bp
RbcL	rbcl_F	5′-TTYATGCGTTGGAGAGAYCG-3′	20 bp
	rbcl_R	5′-GTGCATAGCWCGGTGAATRTG-3′	21 bp
ITS	ITS_F	5′-CTTTCAACAACGGATCTCTTG-3′	21 bp
	ITS_R	5′-TTCAAAGATTCGATGATTCAC-3′	21 bp
16S	16s_F	5′-AGGATGCAAGCGTTATCCGG-3′	20 bp
	16s_R	5′-AATCCCATTCGCTCCCCTAG-3′	20 bp

for 20 s, and 72 °C for 31 s. 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. The amplification efficiency was initially assessed by the slope of the standard curve, with the formula $E = 10^{(-1/slope)}$. Reaction efficiency was also evaluated with LRE analyzer 0.9.10. 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. Assessment of a correct quantification for unknown samples in different template concentrations previously determined by NanoDrop 2000c spectrophotometer was then performed by comparing obtained Ct values against this standard curve.

Fluorescent internal probe 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 StepOnePlusTM Real-Time PCR System instrument (Foster City, CA, USA). Four probes have been designed with the Oligo ArchitectTM 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 on stone substrata and selection of markers

For the first time in this study, a full comprehensive review of molecular data about microorganisms involved in subaerial biofilm formation is presented, digesting over 90 publications regarding biofilm on stone in European sites of interest.

Organisms involved in subaerial biofilm formation represent a huge variety of microalgae, cyanobacteria, fungi, and bacteria (Salvadori and Municchia 2016; Isola et al. 2016). In Fig. 1, geographical distribution of monuments, archeological sites, caves, and buildings of cultural interest across Europe is shown, based on the studies present in cited literature for the compilation of the list of taxa involved in biodeterioration. Due to the extensive collection of identifications from biofilms, we have first tried to point out if some among the listed

Fig. 1 Geographical distribution of sampling sites for biofilm identification regarding stone monuments, caves, and buildings of cultural interest and archeological sites, obtained by the cited case-studies papers

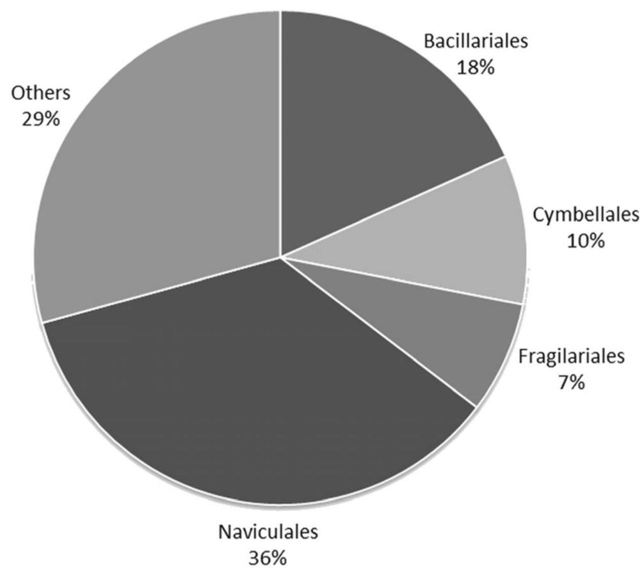


Fig. 2 Percentage of reported diversity of diatoms in the available literature at family taxonomic level

microorganisms were also the principal responsible for the weathering of stone. Unfortunately, the survey could not assess a defined majority that is primarily involved in biological weathering of stone, confirming the idea that settling of microorganisms on stone is a stochastic event.

Diatoms are found free-living in a number of subaerial biofilms where substratum is somehow constantly wet, especially fountains. However, they do not contribute to the great majority of microbial mats on stone. Reported diversity for diatoms (Fig. 2) is great and accounts for a non-negligible part of Naviculales and Bacillariales, followed by 12 other Ordines. Due to the environmental bias, they were excluded by further investigation for marker selection.

Phototrophic bacteria all belong to Cyanobacteria, and their occurrence in subaerial biofilms is almost equally divided among Chroococcales, Nostocales, Oscillatoriales, and Synechococcales (Fig. 3a), thus reflecting their common distribution in the environment. Thanks to their ability to adapt to dim light and retain moisture through massive EPS production, they are well-known pioneers in biofilm establishment (Rossi and De Philippis 2015). Moreover, almost the half of the Genera here collected own a filamentous morphology (Fig. 3e), which enhances surface porosity for the substratum and favors the deposition and embedding of single-cell microorganisms and spore.

On the other hand, eukaryotic green algae are almost all included in the Division of Chlorophyta. Class Trebouxiophyceae, accounting for about the 50% of reported diversity (Fig. 3b), occur as non-flagellate unicells or colonies and sometimes as unbranched or branched filaments in freshwaters and terrestrial environments. Trebouxiophyceae are commonly regarded as a sister group to Chlorophyceae, which in the present study represent about 30% of reported diversity on stone. Green algae morphology is almost equally distributed among filamentous, colonial, and unicellular living forms (Fig. 3e). In presence of water and light, they can show a very fast growing attitude, thus representing a strong driving community in the establishment of a biofilm; however, green algae account for the highest

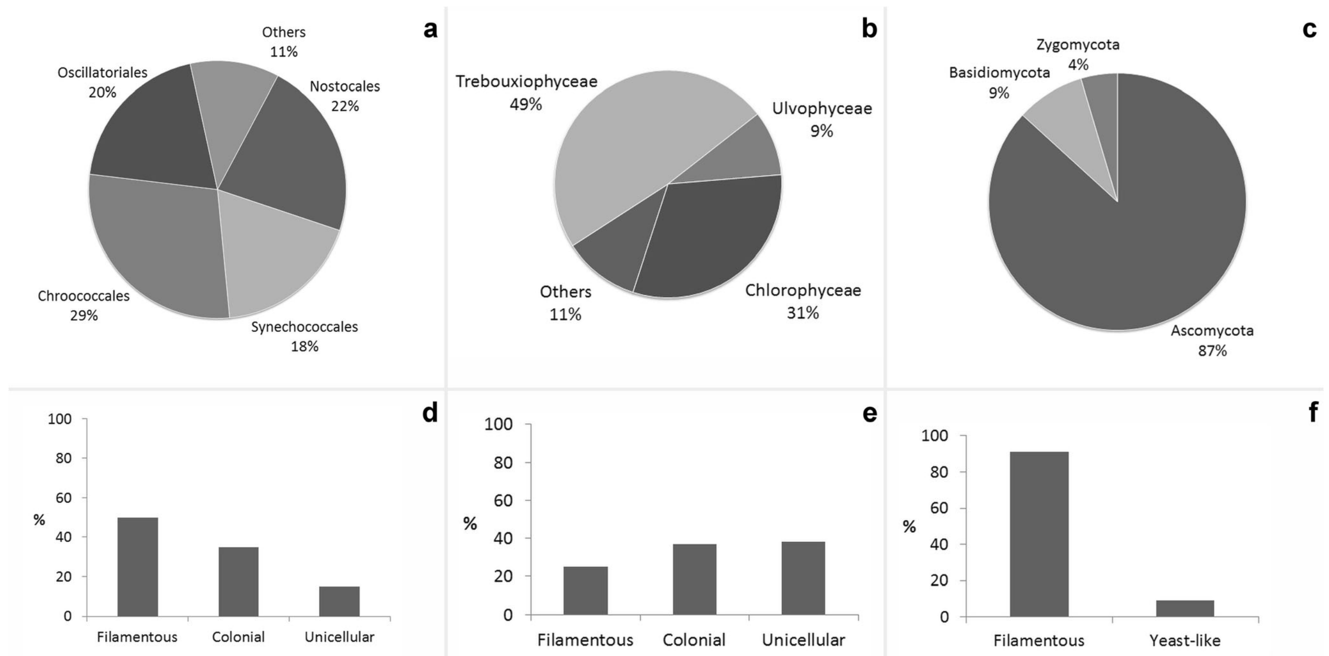


Fig. 3 Percentage of reported diversity of Cyanobacteria (**a**), Chlorophyta (**b**), and Fungi (**c**) in available literature, respectively, at Order, Class, and Phylum taxonomic level. Morphology of retrieved microorganisms is reported in the histograms as percentage of Genera with those features (**d**, **e**, **f**)

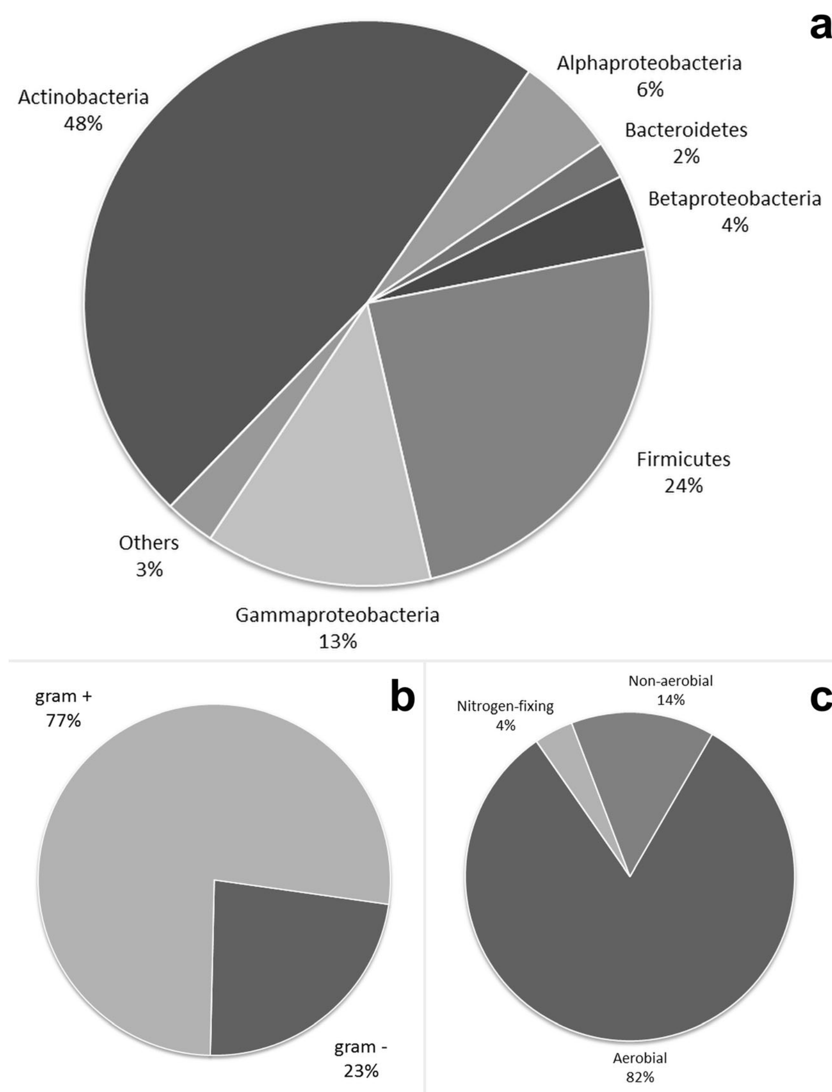


Fig. 4 Percentage of reported diversity for non-phototrophic Bacteria in available literature at Phylum taxononomic level (**a**). Gram stain positivity and preferred metabolism are also reported in percentage in pie charts (**b**, **c**)

taxonomic diversity in this study, with 77 Genera from 313 reports (Supplementary materials Table S3).

Non-phototrophic microorganisms massively involved in subaerial biofilms formation are Fungi and Bacteria. The greatest majority of reported molds is formed from members of Ascomycota Phylum (87%) with a filamentous morphology (Fig. 3c, f), followed by Basidiomycota and Zygomycota. Nevertheless, at a lower taxonomic rank in Ascomycota, Dothideomycetes, Eurotiomycetes, and Sordariomycetes include some of the most retrieved molds in microbial mats, such as *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, etc.

Bacterial growth on stone is somehow less investigated when compared to other microbial groups and taxonomic attribution is often uncertain. Also, VBNC (viable but not culturable) Bacteria are a major issue in biofilm description, since they have been neglected for a long time. Extrapolating data from cited literature, we have found that most of Bacteria involved in subaerial biofilms share three features: (i) they belong to the Actinobacteria or Firmicutes group, (ii) they are positive for Gram staining, and (iii) they have an aerobial metabolism (Fig. 4).

In our survey, we could not find a group of microorganism (taxonomic or metabolic) that is uniformly present across the stone substrata collected by the case-study literature. Rather we have found a wide majority of phototrophic and heterotrophic filamentous microorganisms with a ubiquitous growth attitude. To date, it is still unclear if pioneer colonizers share a common phylogenetic or metabolic trait, except for the ability to survive in very changeable environments with small resources; this may support the idea that morphology is a key feature for the establishment of a novel community on stone substrata (Marasco et al. 2016; Del Mondo et al. 2018). Enhancing surface porosity with network meshes formed by filaments and branches allows the further contribution of single-celled microorganisms and spore, which can secondarily bloom in the biofilm if conditions are appropriate.

In previous years, different techniques have been proposed for the detection and identification bacterial

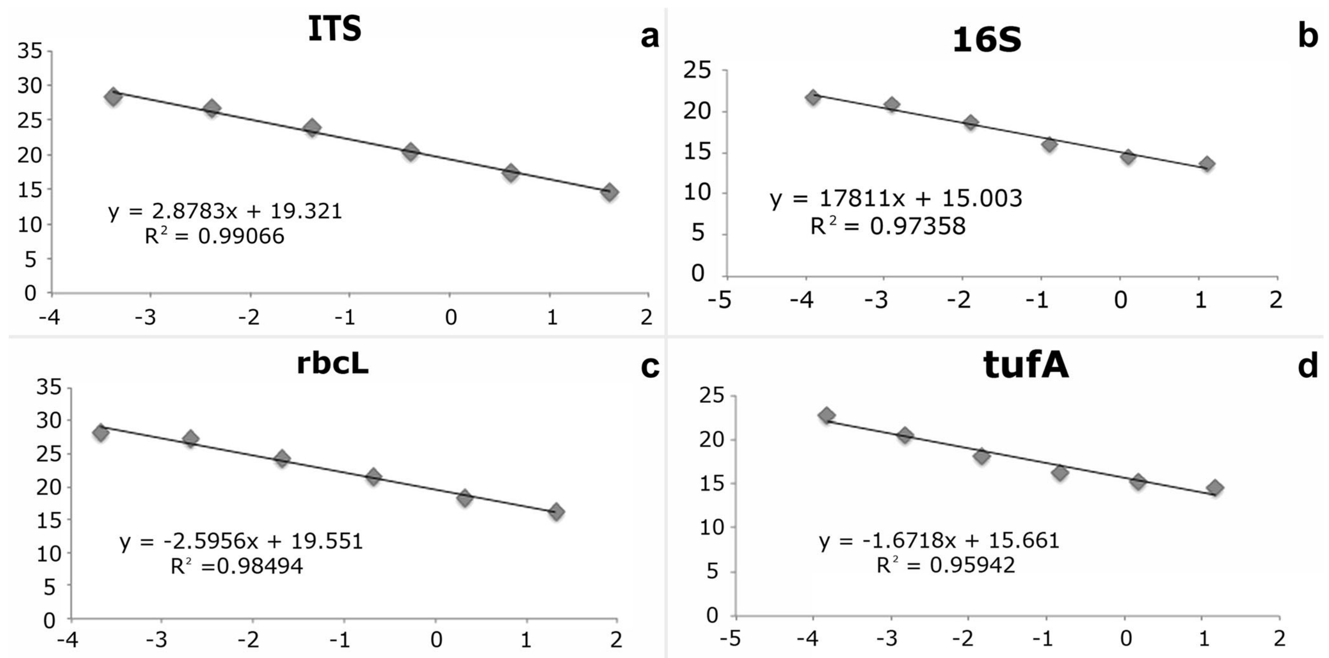


Fig. 5 Standard curves for absolute qPCR quantification for rbcL and tufa barcoding markers for the determination of Chlorophyta and 16S and ITS barcoding markers for the determination, respectively, of Eubacteria and Fungi

communities forming biofilms. The methods use a combination of different techniques, ranging from combination of intracellular fluorochrome and fluorescent-labeled antibody detection (McFeters et al. 1999) to a whole-cell hybridization coupled with laser scanner cytometry (Baudart and Lebaron 2010). When biofilm communities are composed not only by bacteria, but also by fungi and phototrophic organisms, the isolation of DNA from environmental samples followed by direct next-generation sequencing has been attempted (Franklin et al. 2015). Also, barcoding markers are widely used for the identification of microorganisms; nonetheless, their use for the quantification of microorganisms is strictly limited to some particular cases (Pavón et al. 2012) and no suitable primers are available in literature for the specific aim of determining biofilm composition.

When choosing a barcoding marker suitable for a group identification, two major issues need to be taken into consideration: (1) the availability of sequences in the databases and (2) the opportune genetic variability that allows a within-group amplification and the design of internal probes. For these reasons, only four markers by the firstly selected seven were used for qPCR assays, i.e., Rbcl, TufA, 16S, ITS. The chosen barcoding markers responded to the prerequisites of alternation in conserved and variable regions; also, listed microorganisms were broadly represented in GenBank database.

Design proceeded in a way that is discriminating for the three major groups of microorganisms investigated (Eubacteria, Fungi, Green Algae). 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.

Table 2 List of the novel-designed fluorescent internal probes. Fluorochromes 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)	21 bp
ITS1-ITS2	ITS-1Z probe	(FAM) 5′-TAG[+C]AAA[+G]T[+G][+C][+G]AT[+A]A[+C]TAG-3′ (BHQ-1)	20 bp
ITS1–1752	ITS-2A probe	(FAM) 5′-CAGCG[+A][+A][+A][+T][+G][+C][+G][+A]TAAGTAA-3′ (BHQ-1)	20 bp
16S	16S-eubat probe	(TAMRA) 5′-GTGTAGCG[+G]T[+G]AAATGCGTAG-3′ (BHQ-2)	21 bp

Standard qPCR curves from mixed templates

To determine the sensitivity of the real-time PCR system developed, standard curves relating Ct values and the logarithm of DNA were built (Fig. 5). The slopes of the linear equations were considered not reliable (> 100%). It is reported that overestimation in reaction efficiencies may indicate pipetting errors or contaminations (González-Salgado et al. 2009). However, it is the first time in which a standard curve is derived by mixed microbial DNA 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 validated by indirectly quantifying mixed DNA specimens from in vitro experiments (*data not shown*).

Improving the sensitivity with internal fluorescent probes

Multiplexing is a technique in which more than one target is analyzed in the same real-time PCR reaction. Each target is distinguished by a particular dye, conjugated to the fluorogenic specific for that target. Peculiar benefits of multiplexing are increased throughput (more samples potentially assayed per plate), reduced sample usage, and reduced reagent usage. The precision benefit of multiplexing is especially valuable for quantitative experiments requiring a higher degree of precision. Here, four probes are presented (Table 2) to be used for a general characterization of subaerial biofilms. Each probe has been designed to fit a group-specific variable region within a short amplicon for each selected barcoding marker. LNAs have been inserted in order to increase melting temperature of the probes (Decousser et al. 2006), which is critical for the accuracy in recognizing the correct template region. Primer and probe design is arguably the most critical factor in a multiplex assay. However, as reaction complexity increases, so does the probability that primers and probes will dimerize or that competition for reaction components will limit the amplification of one or more targets. In order to prevent these complications, BLAST searches and in silico simulations have been performed for each combination of primer and probe to ensure the specificity for the targets of interest. A major issue in the use of barcode marker amplification with internal fluorescent probes is that collected template sequences which differ from the known sequence for the phylo-group may not be detected; this could affect the quantification of the true sampled population. Nevertheless, a way to overcome this problem is to design cocktails of probes which include as much microbial diversity as possible, also thanks the everlasting efforts of taxonomists collecting and characterizing biofilm forming microorganisms.

Conclusions

In recent times, molecular biology techniques have been successfully applied in order to understand composition and structure of microbial communities, avoiding the cultivation and the isolation of single components. Estimates of microbial composition, diversity, and even ecological interactions are performed using a variety of culture-independent approaches including metagenomics (McLean and Kakirde 2013; Pfendler et al. 2018); however, in this study, we propose an approach for the characterization of subaerial biofilms which is intermediate between metagenomic analysis and culture-dependent methods for species identification.

In most cases, the diagnosis of microbial communities established on stone surfaces of valuable interests does not require a punctual description of microorganisms involved, despite of the cutting-the-edge used technology. Here, we propose a novel system aimed for a fast and cost-effective evaluation of the microbial community on the basis of its main actors for the understanding of the establishment and development of subaerial biofilms. The proposed approach employs qPCR primers and fluorescent internal probes for the characterization and quantification microbial groups of interest in a biofilm. Real-time PCR (qPCR) is an extremely sensitive assay, which allows the quantification of few copies of target DNA; if coupled with internal fluorescent probes, it can also be informative about differential targets within the template, i.e., groups of phylogenetically distinct microorganisms or groups. 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 cost-effective approach in the study of biofilms for the estimation of algae, molds, and bacteria both for direct in situ analysis and in vitro simulation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A. This research did not involve human participants and/or animals.

Informed consent N/A. This research did not involve human participants.

References

Agrawal PK, Agrawal S, Shrivastava R (2015) Modern molecular approaches for analyzing microbial diversity from mushroom compost ecosystem. 3 Biotech 5:853–866

Baudart J, Lebaron P (2010) Rapid detection of Escherichia coli in waters using fluorescent in situ hybridization, direct viable counting and solid phase cytometry. J Appl Microbiol 109:1253–1264

Caneva G, Nugari MP, Salvadori O (Eds.) (2008) Plant biology for cultural heritage: biodeterioration and conservation. Getty Publications.

Dakal T, Arora P (2012) Evaluation of potential of molecular and physical techniques in studying biodeterioration. Rev Environ Sci Biotechnol 11:1–34

Decousser JW, Methlouthi I, Pina P, Collignon A, Allouch P (2006) New real-time PCR assay using locked nucleic acid probes to assess prevalence of ParC mutations in fluoroquinolone-susceptible *Streptococcus pneumoniae* isolates from France. Antimicrob Agents Chemother 50:1594–1598

Del Mondo A, Pinto G, Carbone DA, Pollio A, De Natale A (2018) Biofilm architecture on different substrates of an Oculatella subterranea (cyanobacteria) strain isolated from Pompeii archaeological site (Italy). Environ Sci Pollut Rer Int 25(26):26079–26089

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Du G, Wu F, Mao Y, Guo S, Xue H, Bi G (2013) DNA barcoding assessment of green macroalgae in coastal zone around Qingdao, China. J Ocean Univ China 13(1):97–103

Engels B (2015) University of Wisconsin. https://engels.genetics.wisc.edu

Franklin MJ, Chang C, Akiyama T, Bothner B (2015) New technologies for studying biofilms. Microbiol Spectr 3(4)

Gonzalez JM, Saiz-Jimenez C (2005) A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. Extremophiles 9(1):75–79

González-Salgado A, Patiño B, Gil-Serna J, Vázquez C, González-Jaén MT (2009) Specific detection of Aspergillus carbonarius by SYBR® green and TaqMan® quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. FEMS Microbiol Lett 295(1):57–66

Gorbushina AA (2007) Life on the rocks. Environ Microbiol 9(7):1613–1631

Hall JD, Fucikova K, Lo C, Lewis LA, Karol KG (2010) An assessment of proposed DNA barcodes in freshwater greenalgae. Cryptogam Algol 31(4):529–555

Isola D, Zucconi L, Onofri S, Caneva G, De Hoog GS, Selbmann L (2016) Extremotolerant rock inhabiting black fungi from Italian monumental sites. Fungal Divers 76(1):75–96

Marasco A, Nocerino S, Pinto G, Pollio A, Trojsi G, De Natale A (2016) Weathering of a Roman mosaic—a biological and quantitative study on in vitro colonization of calcareous tesserae by phototrophic microorganisms. PLoS One 11(10):e0164487

Miller TR, McMahon KD (2011) Genetic diversity of cyanobacteria in four eutrophic lakes. FEMS Microbiology Ecology 78 (2):336-348

McFeters GA, Pyle BH, Lisle JT, Broadway SC (1999) Rapid direct methods for enumeration o specific, active bacteria in water and biofilms. Soc Appl Bacteriol Symp Ser 85:193S–200S

McLean RJ, Kakirde KS (2013) Enhancing metagenomics investigations of microbial interactions with biofilm technology. Int J Mol Sci 14(11):22246–22257

Neilan BA, Jacobs D, Goodman AE (1995) Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. Appl Environ Microbiol 11(61):3875–3883

Neilsona JW, Jordan FL, Maier RM (2013) Analysis of artifacts suggests DGGE should not be used for quantitative diversity analysis. J Microbiol Methods 92(3):256–263

Okonechnikov K, Golosova O, Fursov M, the UGENE team (2012) Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28:1166–1167

Op De Beeck M, Lievens B, Busschaert P, Declerck S, Vangronsveld J, Colpaert JV (2014) Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. PLoS One 9(6): e97629

Pavón MA, González I, Martín R, García Lacarra T (2012) ITS-based detection and quantification of Alternaria spp. in raw and processed vegetables by real-time quantitative PCR. Food Microbiology 32 (1):165-171

Pfendler S, Karimi B, Maron PA, Ciadamidaro L, Valot B, Bousta F, Alaoui-Sosse L, Alaoui-Sosse B, Aleya L (2018) Biofilm biodiversity in French and Swiss show caves using the metabarcoding approach: first data. Sci Total Environ 615:1207–1217

Poly F, Jocteur Monrozier L, Bally R (2001) Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Res Microbiol 152:95–103

Rastogi G, Sani RK (2011) Molecular techniques to assess microbial community structure, function and dynamics in the environment. In: Ahmad I (ed) Microbes and microbial technology. Springer, Berlin, pp 29–57

Roeselers G, van Loosdrecht MCM, Muyzer G (2008) Phototrophic biofilms and their potential applications. J Appl Phycol 20(3):227–235

Rossi F, De Philippis R (2015) Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial Mats. Life 5:1218–1238

Rutledge RG (2011) A Java program for LRE-based real-time qPCR that enables large-scale absolute quantification. PLoS One 6(3):e17636

Rutledge RG, Stewart D (2008) A kinetic-based sigmoidal model for the polymerase chain reaction and its application to high-capacity absolute quantitative real-time PCR. BMC Biotechnol 8:47

Salvadori O, Municchia AC (2016) The role of fungi and lichens in the biodeterioration of stone monuments. The Open Conference Proceedings Journal 7(suppl 1: M4):39–54

Saunders GW, Kucera H (2010) An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. Cryptogam Algol 31(4):487–528

Scheerer S, Ortega-Morales O, Gaylarde C (2009) Microbial deterioration of stone monuments - an updated overview. In: Laskin AL, Saraslani S, Gadd G (eds) Adv Microbiol 66:97–139

Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. Nature Reviews Microbiology 6 (3):199-210

Stone M (2015) Small Talk: The Evolution of Bacterial Languages. BioScience 65 (3):336-336

Tiano A, Carreras, M, Ridao P, Zirilli A (2002) On the identification of non linear models of unmanned underwater vehicles. In: 10th Mediterranean Conference on Control and Automation 9–12, Lisbon, Portugal

Tolker-Nielsen T, Molin S (2000) Spatial organization of microbial biofilm communities. Microb Ecol 40:75–84

Tomaselli L, Tiano P, Lamenti G (2000) Occurrence and fluctuation in photosynthetic biocoenoses dwelling on stone monuments. In: Of microbes and art – The role of microbial communities in the degradation and protection of cultural heritage, pp. 63–76.

Urzì C (2004) Microbial deterioration of rocks and marble monuments of the Mediterranean basin: a review. Corros Rev 22(5–6):441–458

Viles HA, Cutler NA (2012) Global environmental change and the biology of heritage structures. Glob Chang Biol 18:2406–2418

Warscheid T, Braams J (2000) Biodeterioration of stone: a review. Int Biodeterior Biodegradation 46:343–368

Zakharova YR, Galachyants YP, Kurilkina MI, Likhoshvay AV, Petrova DP (2013) The structure of microbial community and degradation of diatoms in the deep near-bottom layer of lake Baikal. PLoS One 8(4):e59977

Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. Appl Environ Microbiol 55(10):2522–2526. https://aem.asm.org/content/aem/55/10/2522.full.pdf

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.