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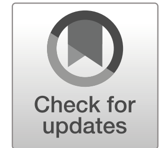

Survey of relevant taxonomic groups for the design of qPCR primers and internal fluorescent probes for whole characterization of subaerial biofilm

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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.

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. The aim of the present study was 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.

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 · Biofilm · Quantitative determination · Biodeterioration · 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. 2000a, b, c). 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 populations (Roeselers et al. 2008). In this case, organic substrates

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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 is described a novel approach for the characterization of subaerial biofilm by focusing on the universal components in epilithic communities in order to understand the role of metabolic drivers in biofilm establishment and development; also, the novel approach could contribute for an easier correlation between biofilm composition and degree of deterioration of stone monuments.

A novel approach to characterize subaerial biofilms

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 alga 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. The aim of the present study is to describe the role of microorganism involved in biofilm formation in European 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, 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). 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 were 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 $MgCl_2$, 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 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 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, and ITS (Table 1). 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 (Supplementary S6). The PCR reactions were carried out using the RealAmp™ 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 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. Reaction efficiency was evaluated with LRE analyzer 0.9.10.

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 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 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, soil fungi, and bacteria (Salvadori and Municchia 2016; Isola et al. 2016). Figure 1 shows geographical distribution of monuments, archeological sites, caves, and buildings of cultural interest across Europe, based on the studies present in cited literature for the compilation of the list of taxa involved in biodeterioration. Our 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

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	3′-GTGCATAGCWCGGTGAATRTG-3	21 bp
ITS	ITS_F	5′-CTTTCAACAACGATCTCTTG-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

Diatoms (Fig. 2) is great and accounts for a non-negligible part of Naviculales and Bacillariales, followed by 12 other Families. 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 natural 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. Trebouxiophyceae,

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-study papers

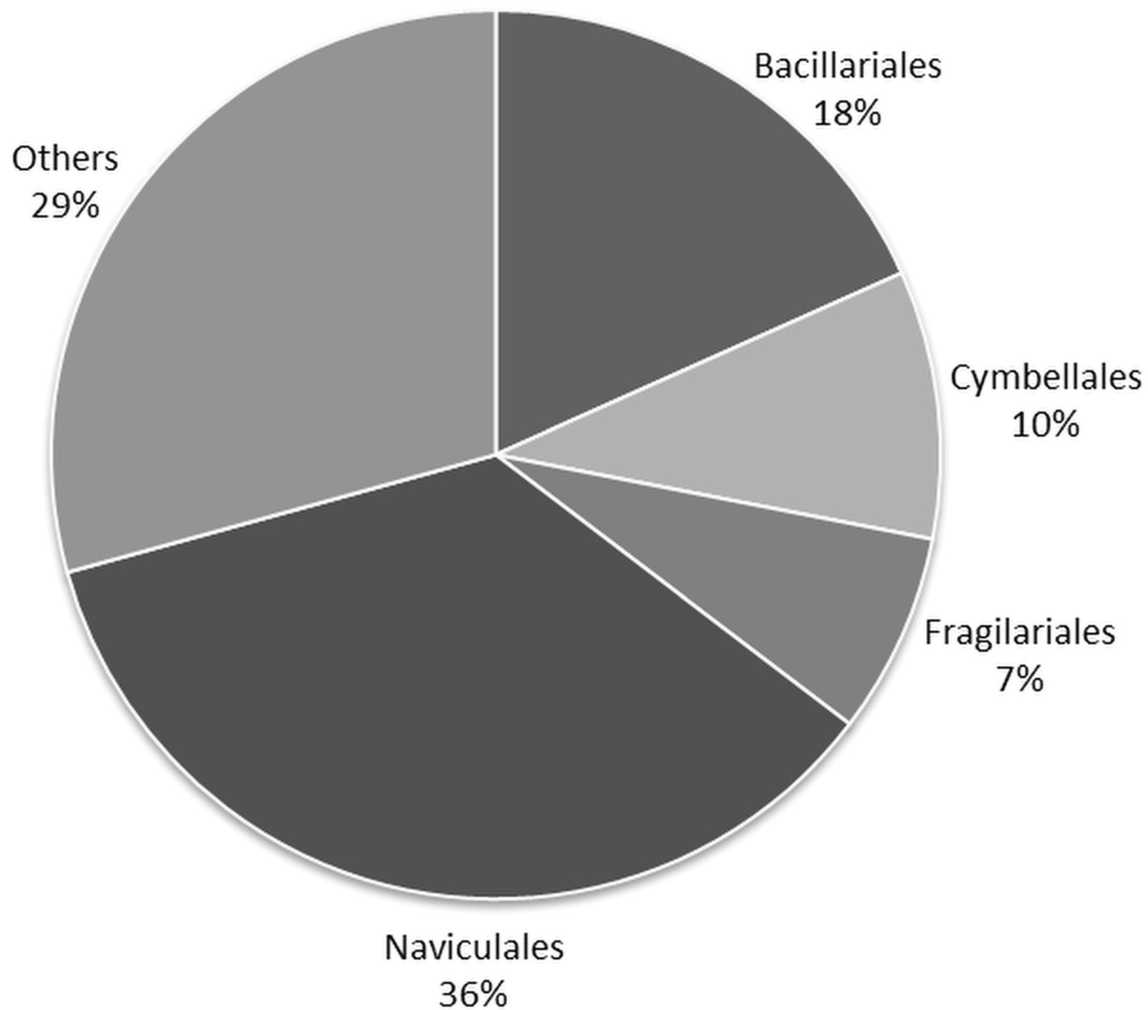

Fig. 2 Percentage of reported diversity of Diatoms in the available literature at Family taxonomic level

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 alga 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 taxonomic diversity in this study, with 77 Genera from 313 reports (Supplementary material 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*, and *Fusarium*.

Bacterial growth on stone is somehow less investigated 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, namely (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).

The fact that no precise involvement of one or more species is necessary in biofilm formation 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); moreover, the participation of ubiquitous soil and freshwater microorganisms occurs in a way that could be primarily influenced by environmental parameters or metabolic features. Enhancing surface porosity with network

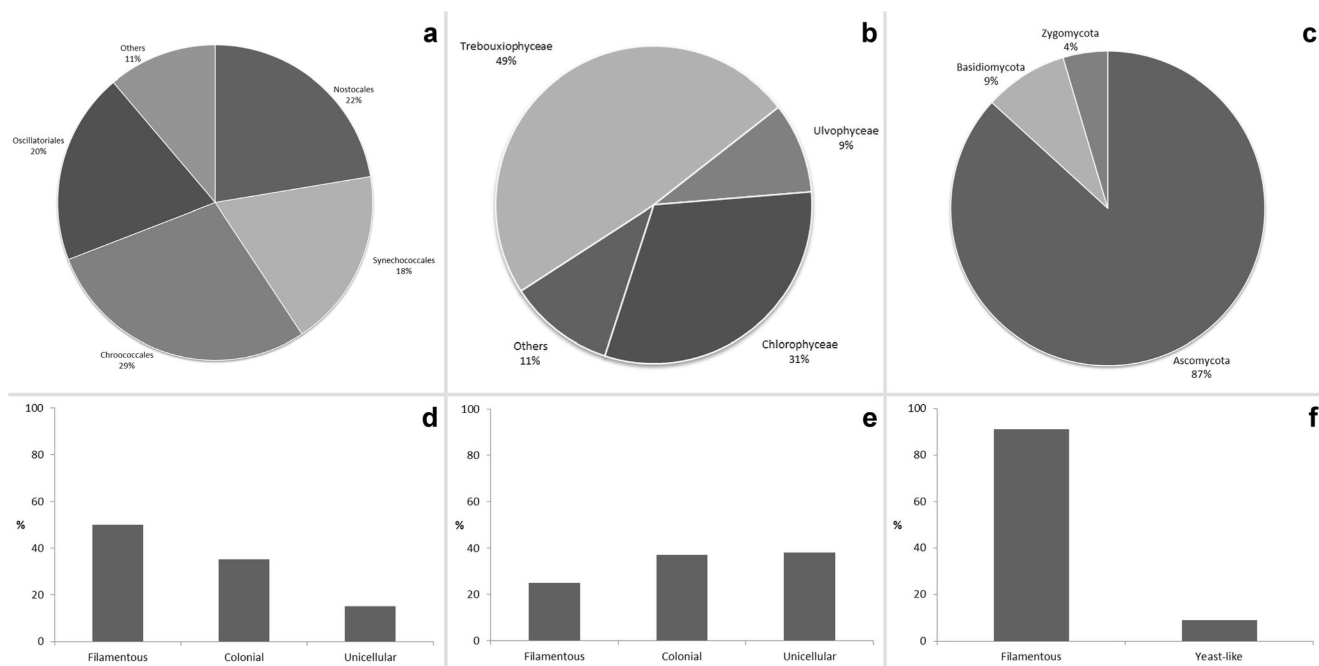

Fig. 3 Percentage of reported diversity of Cyanobacteria (**a**), Chlorophyta (**b**), and Fungi (**c**) in available literature, respectively at Order, Class, and Phylum taxonomic level. Morphology of retrieved microorganisms is reported in the histograms as percentage of Genera with those features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

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.

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 (Pavon 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 needs 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, and 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. 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.

Standard qPCR curves from mixed templates

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 is, the lower the Ct value for that reaction is. 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 amplification efficiency was initially assessed by the slope of the standard curve, with the formula $E = 10^{(-1/\text{slope})}$. 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 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 used to indirectly quantify mixed DNA specimens from in vitro experiments (*data not shown*).

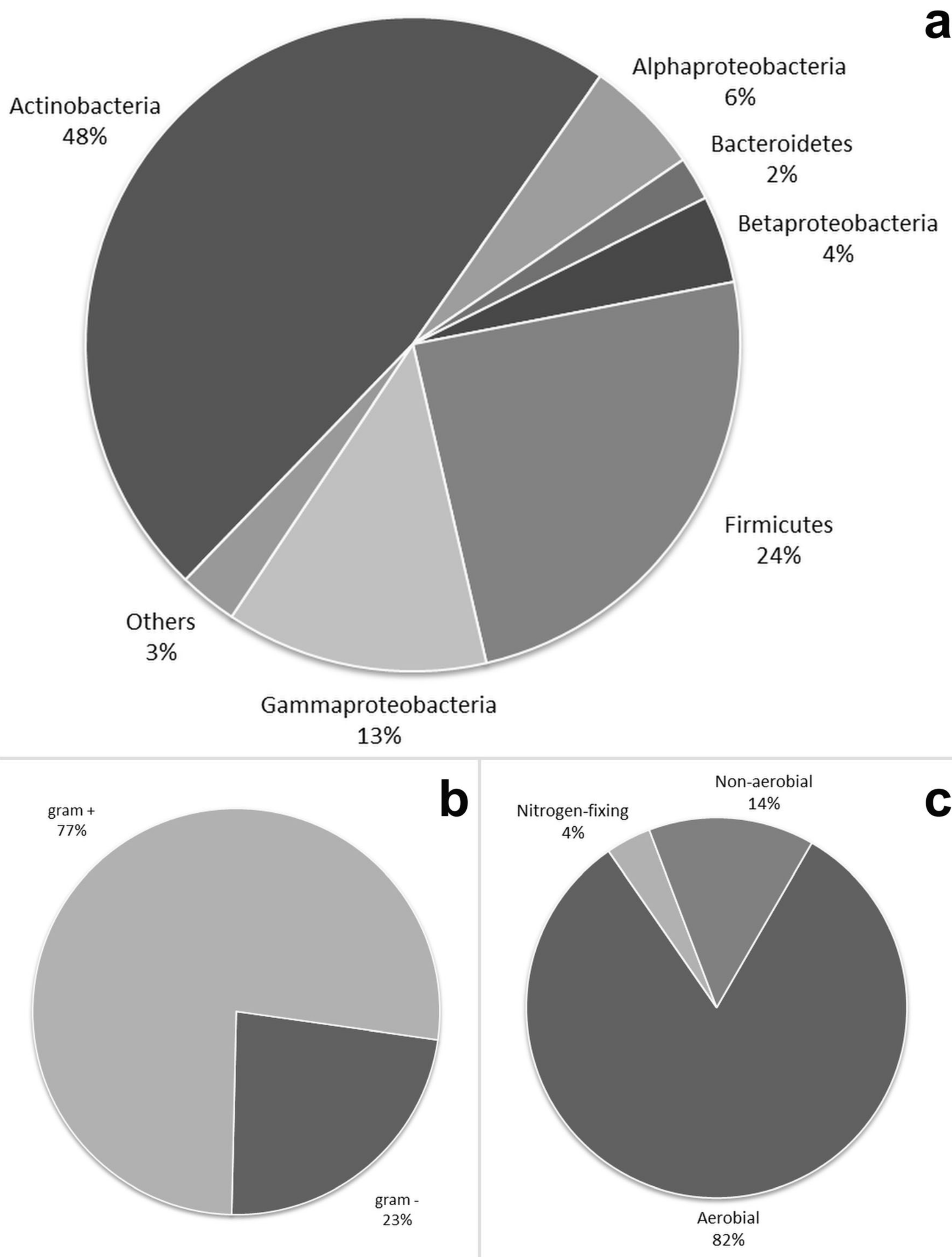

Fig. 4 Percentage of reported diversity for non-phototrophic Bacteria in available literature at Phylum taxononomic level (**a**). Gram stain positivity and preferred metabolism are also reported in percentage in pie charts (**b**, **c**).

Improving the sensitivity with internal fluorescent probes

Profiling microbial mats through the quantification of the metabolic drivers 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

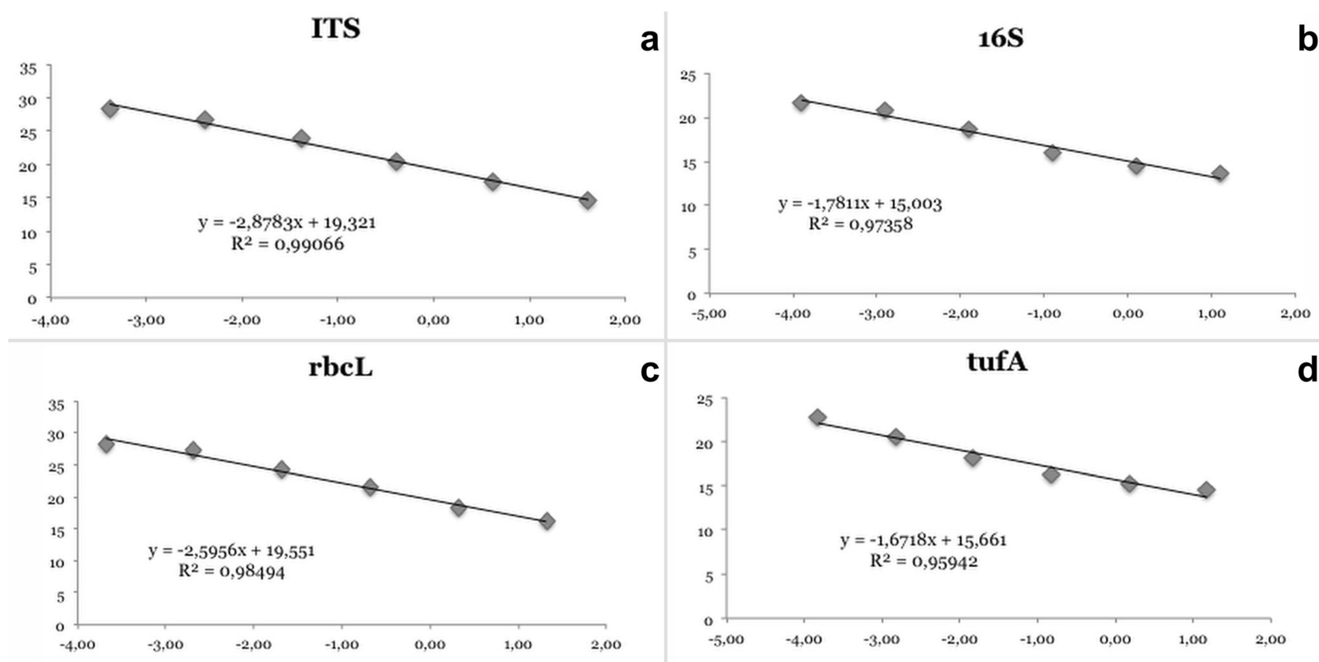

Fig. 5 Standard curves for absolute qPCR quantification for rbcL and tufa barcoding markers for the determination of Chlorophyta and 16S and ITS barcoding markers for the determination, respectively, of Eubacteria and Fungi

Table 2 List of the novel-designed fluorescent internal probes

Maker	Oligo name	Sequence	Length
Tufa	Tufa probe	(JOE)5′-YTTAAATA[+A][+A][+G][+A]AGAYCAAGT0–3′(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-ITS2	ITS-I2A 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

Fluorochromes and quenchers were chosen to be compatible in a multiplex reaction. Letters in square brackets symbolize LNA nucleotides

metabolic groups, offering new insights for biofilm characterization, such as monitoring of biodeterioration, determination of ecological successions in biofilms, and assessment of biocide efficiency. The high specificity and sensitivity of the probes require small amounts of sample, fournishing results in a very small amount of time. Here four probes are presented (Table 2) to be used for a general characterization of subaerial biofilms.

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 fact, more than a deep description of all the species involved in a mat, often represented by very few individuals, an evaluation of the microbial community on the basis of its main actors may be a useful tool to deeply understand establishment and development of subaerial biofilms. In this work we presented a novel approach for the study of subaerial biofilms through the employment 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 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.

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Compliance with ethical standards

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

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

Informed consent This research did not involve human participants.

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