FRESHWATER CYANOBACTERIA, IDENTIFIED BY MICROSCOPIC AND MOLECULAR INVESTIGATIONS ON A COLONIZED FOUNTAIN SURFACE: A CASE STUDY IN PALERMO (SICILY, ITALY)

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1. Introduction

Cyanobacteria, more commonly known with the name of blue or blue-green algae (Cyanophyceae), are a heterogeneous group of single-cell photoautotrophic organisms [1]. They are the only group of prokaryotic algae, and it has been ascertained that they were the first organisms to produce organic matter on earth and the first photosynthetic organisms to release oxygen into the atmosphere [2]. They are widespread in several habitats with various ecological conditions, being able to develop both in marine and terrestrial ecosystems, and also in extreme environments such as glaciers, deserts, marine habitats and freshwater, and rocks or sediments [3]. Cyanobacterial growth and replication time depend on environmental factors such as temperature (optimal range being 35-40°C), light, water, carbon dioxide and inorganic substrates, except for some species that are able to survive in complete darkness [4]. Most cyanobacteria are autotrophic aerobic organisms, but some show a distinct ability for heterotrophic nutrition, and some are anaerobic. These organisms are of different sizes, ranging from microscopic to macroscopic and have a coccoid or filamentous shape. Among the filamentous species there are some that develop particular cell types, called heterocysts, surrounded by a very thick wall, which depend on other cells for nourishment and performing atmospheric nitrogen fixation. Many filamentous cyanobacteria also develop a second cell type called akinetes, similar to the spores that germinate when there are favourable conditions for growth [3]. Their photosynthetic activity, similar to that of higher plants, contain pigments such as chlorophyll-a, phycoerythrin, carotenoid, xanthophylls and phycocyanin [3]. Phycobiliproteins are responsible for the characteristic blue-green colouring of cyanobacteria and are able to absorb visible light with a wavelength ranging from 500 to 650 nm, in order to perform photochemical reactions [5].

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The classification of cyanobacteria has been, and still is, the subject of numerous reviews both from a bacteriological and botanical point of view [6]. The most common classification code is based on their morphological and cytological characteristics, by which the Cyanophyceae class is divided into four orders: Chroococcales, Oscillatoriales, Nostocales, Stigonematales [7-8].

Cyanobacteria have potential application in the field of human healthcare as they are known to produce a variety of bioactive compounds. Therefore, it may be possible to hypothesize their potential use in many applications in the fields of bioenergy, natural products, medicine, agriculture, and the environment [9-10]. On the contrary, the spread of cyanobacteria in fresh water represents an increasing problem related to the quality of surface water. In fact, the excess supply of nutrients (phosphorus and nitrogen) determines the phenomenon of water eutrophication. In particular, some species belonging to the genera Oscillatoria, Microcystis, Anabaena, are able to form large blooms on the surface of rivers, lakes and seas, known by the term water bloom [3]. Cyanobacteria are able to produce toxic substances (cyanotoxins), representing a risk for human health [11] and acting on many ecosystems. They are also responsible for the large number of deaths of fish, crustaceans and other animals [12]. Frequently, cyanotoxins such as microcystins, nodularins, cylindrospermopsin, Lyngbya toxins, anatoxins can be present. Toxicosis from marine phycotoxin producers in animals can occur from direct contact via skin, inhalation or ingestion of toxigenic organisms, or by ingestion through the food chain. The effects of different phycotoxins range from respiratory paralysis to acute liver or nervous system damage, skin irritation and tumor promotion [13].

Moreover, cyanobacteria form an external mass of mucilaginous material, which serves as protection for the structure (biofilm) that holds the microbial cell colonies together, allowing them to adhere to the substrate, inducing biodeterioration processes [14]. Both macro-organisms (animals, plants) and microorganisms (bacteria, fungi, cyanobacteria, algae and lichens) can trigger the biodeterioration of cultural assets [15]. Identifying the biological systems on artwork surfaces is essential to accurately evaluate biodeterioration processes, perform proper sampling and integrated investigations based on microscopy, *in vitro* microbial culture and molecular biology analysis [16].

The aim of this study was to reveal and identify, through an integrated approach (microscopy analysis and molecular biology investigation), cyanobacteria colonies in the complex biofilm colonizing the surface of the marble fountain of the Two Dragons (Fontana dei Due Draghi) in Palermo city centre (Sicily, Italy). In particular, combining optical microscopy analysis, in bright and fluorescence fields, and molecular biology investigation by quantitative Polymerase Chain Reaction (qPCR), information on the presence and amount of cyanobacteria colonies was gathered. Specifically, *Thioredoxin reductase* protein was utilized to perform the corresponding phylogenetic tree of plants, animals and cyanobacteria, showing that it can be a good molecular marker for the identification of cyanobacteria colonies.

2. Materials and methods

2.1. Microscopy analysis

Biofilm samples were taken from selected areas of a historical 17th century marble fountain and stored (in water at room temperature and exposed to the daily light cycle) until their use for investigation.

Micro-fragments were directly observed with an Axioscop2 plus microscope (Zeiss, Jena, Germany) and images acquired using a Zeiss Axiocam camera (Zeiss 412-312, magnification of 20X, 40X and 60X), in bright and fluorescence fields. The excitation and emission filter sets were:

i) blue light excitation with emission in green (BP450-490/FT510/LP520; FITC, Fluorescein isothiocyanate), exciting chlorophyll-a (which appears red) and phycoerythrin type I (which appears yellow-orange);

ii) green light excitation with emission in yellow/orange (BP520-560/FT580/LP590; TRITC, Tetra-methyl-rhodamine), exciting both phycocyanin and phycoerythrin type II, emitting fluorescence in red and orange-red respectively.

2.2. Extraction of genomic DNA

Biofilm fragments were first dehydrated (keeping them between absorbent paper sheets for two hours), then removed and pulverized by mortar and pestle in the presence of liquid nitrogen; 200 ng of this powder were used for extraction of the genomic DNA (gDNA) by GeneElute Blood Genomic DNA Kit (Sigma Aldrich, St Louis, MO, USA), following the instructions in the technical manual. The RNA-free DNA (RNase-A incubation for 20 min at 37°C) was quantified using a Bio-photometer D30 (Eppendorf, Hamburg, Germany) at 260 nm (1 O.D. equal to 50 ng/μL). Aliquots of gDNA (20 ng) were the template molecules in Real-Time qPCR reactions.

2.3. Real-Time quantitative PCR (qPCR)

The quantitative analysis of the genomic DNA target sequences was carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's manual. A comparative threshold cycle method with SYBR Green master mix was performed [17]. To perform the qPCR, the following parameters were used: a denaturation cycle at 95 ° C for 10 minutes to allow activation of the polymerase; 38x cycles: melting at 95 ° C for 15 seconds, annealing/extension at 60 ° C for 60 seconds. The *16S-rRNA* was the housekeeping gene, while the *Thioredoxin reductase* (TrxR) was the target gene. DNA amplification was performed by specific primers for *16S-rRNA* and TrxR coding genes. For the *16S-rRNA* gene, primers were: Forward = 5'-AGTAGCTGGTCTGAGAGGATGA-3' and Reverse = 5'-CGGTATTGCTCCGTCAGGCTTTC-3'; amplicon was 118 bp in length. The primers specific for the TrxR target sequence were: Forward = 5'-AAGGCTTGGAGTGGGATGGCGAA-3 'and Reverse = 5'- TGCCACAGATACTTGATTCAAGCC -3 '; amplicon size was 116 bp in length.

Results are the average of two qPCR experiments.

2.4. Phylogenetic analysis of Thioredoxin reductase proteins

TrxR proteins sequences from different organisms, cyanobacteria, vertebrates and invertebrates, were analysed using the BLAST program (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/); their accession numbers are indicated in Table 1. Scientific research produces a large volume of complex data, which is collected and made available in databases that represent peculiar sources of information on different

biological molecules (DNA, RNA, Protein), very frequently used in science. In this case study, the Genbank accession numbers are related to Thioredoxin reductase protein sequences, allowing the identification of macro or micro biological species. Specifically, multiple sequence alignments were performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the evolutionary analysis was conducted with MEGA-X software [18] we report a transformation of MEGA to enable cross-platform use on Microsoft Windows and Linux operating systems. MEGA X does not require virtualization or emulation software and provides a uniform user experience across platforms. MEGA X has additionally been upgraded to use multiple computing cores for many molecular evolutionary analyses. MEGA X is available in two interfaces (graphical and command line, using the Neighbour-Joining (NJ) method [19].

The ExPASy proteomic server (http://www.expasy.org/) was used to perform L-Align, in order to find the similarity percentage among TrxR proteins.

Table 1. Thioredoxin reductase proteins Genbank accession numbers and organisms used in the phylogenetic analysis. The percentage of similarity refers to Cyanobacterium sp.

Organisms	Genbank Accession number	Percentage of similarity (%) / number of amino acids
Cyanobacterium sp.	AFZ46444.1	
Synechococcus sp.	MBF2080429.1	97/328
Oscillatoria sp.	TAF50584.1	96/328
Rivularia sp.	WP_015121190.1	96/329
Leptolyngbya sp.	WP_075598049.1	96/328
Synechocystis sp.	WP_066342850.1	97/328
Homo sapiens	AAB35418.1	58/112
Drosophila melanogaster	NP_524216.1	58/190
Bacillus sp.	KRF49594.1	57/331
Arabidopsis thaliana	CAA80656.1	53/308
Chlorella vulgaris	BAH29954.1	55/332

2.5. Statistical analysis

The statistical analysis of the qPCR results was performed using the ANOVA one-way analysis of variance test, with R statistical software (http: //www.r-project.org, Vienna, Austria) and significance at $p < 0.05$, followed by Tukey's multiple comparison test.

3. Results

3.1. Microscopy analysis

In order to evaluate the presence of cyanobacteria, which are potentially toxic for human and animal health and /or harmful for cultural assets, pigmented green biofilms were collected from the marble fountain surface, as shown in Figure 1A (indicated by the white square).

Tiny fragments of biofilm were observed under optical microscope as described in Materials and Methods by seeing them in bright and fluorescence fields (Figure 1 and 2), taking advantage of the fact that cyanobacteria naturally emit fluorescence (in green and red), as they have pigments such as chlorophyll, carotenoid and phycobilin-proteins exhibiting different colours [20].

In Figure 1B the filamentous *Oscillatoria sp.*, *Lyngbya* sp. and the coccoid cyanobacteria *Chroococcus* sp., surrounded by other unidentified organisms, were observed.

Figure 1C shows a wide field of *Gloeocapsa sp.,* a unicellular colonial alga whose colonies are small and irregularly aggregated and enclosed in a mucilaginous matrix; it is sometimes found covering large areas of wet stony substrates. It counts many species as reported by the Taxonomic outlines of Bergey's Manual of Systematic Bacteriology [21] and counts 130 species as reported by AlgaeBase (a global algal database, available at: https://www.Algaebase.org).

Figure 1D highlights the presence of cyanobacteria, such as *Chroococcus* sp. and the tuft of a filamentous branching *Dichothrix* sp.*,* in particular, which is clearly recognizable in the red fluorescence field.

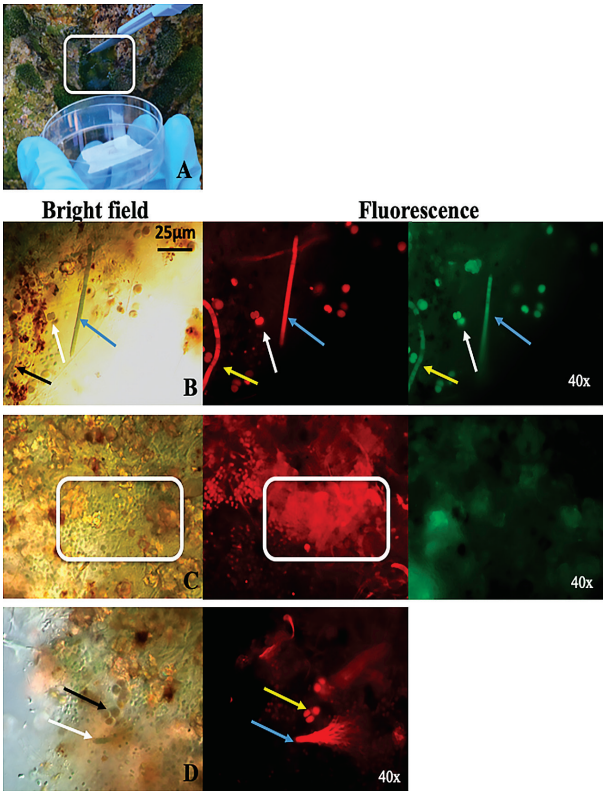

Figure 1. Sampling of the Two Dragons biofilm. A) Due to their autofluorescence (red and green), microscopy analysis in the bright and fluorescence fields highlights several cyanobacteria morphologies, indicated by coloured arrows; B) black arrow: Lyngbya sp; white arrow: Chroococcus sp.; light blue arrow: Oscillatoria sp.; yellow arrow: Lyngbya sp.; C) white square: Gloeocapsa sp.; D) black and yellow arrows: Chroococcus sp.; white and light blue arrows: Dichothrix sp.

In Figure 2A, the *Oscillatoria sp.* cyanobacteria is recognizable. Since hundreds of species are attributable to the *Oscillatoria* genus, as recorded in the AlgaeBase database, correct identification of the species is quite difficult. The filamentous cyanobacterium *Microcoleus* sp. is shown in Figure 2B. The filament of this genus is composed of a gelatinous colourless sheath, in which are packed parallel trichomes, the number of which usually changes from 2 to more than 100 within one sheath. Figure 2C shows a colony of *Chroococcus turgidus*, located in the biofilm edge, in red and green fields. They are spherical unicellular or colonial organisms, usually few-celled, forming a gelatinous biofilm [22]. Figure 2D shows the sheath of the cyanobacteria *Lyngbya* sp.

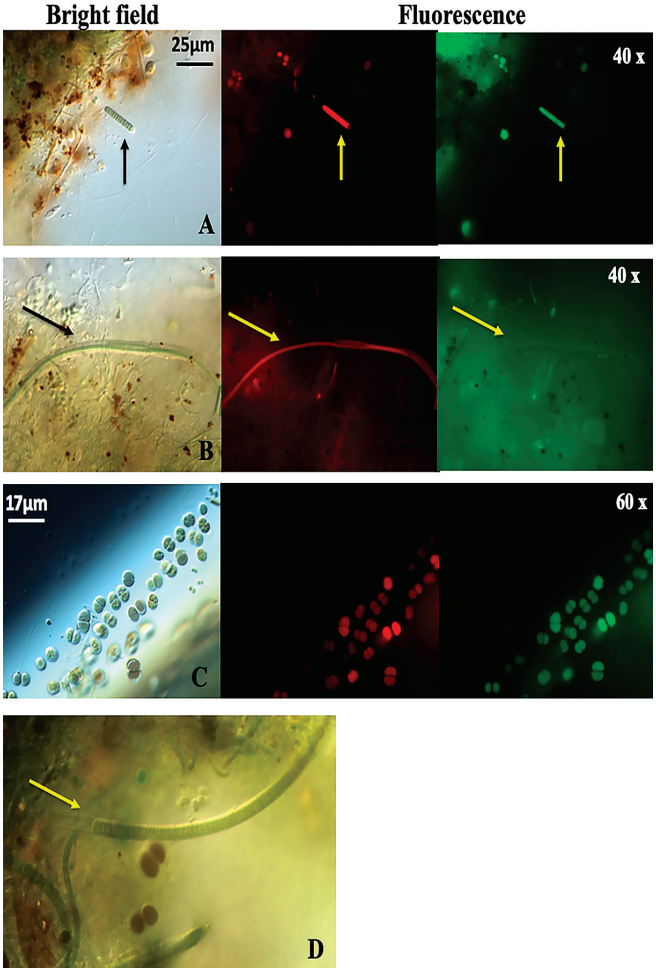

Figure 2. Morphological profiles in bright and fluorescence fields (red and green) of cyanobacterial species revealed in the fountain biofilm. A) black and yellow arrows: Oscillatoria sp.; B) black and yellow arrows: Microcoleus sp.; C) Chroococcus turgidus; D) yellow arrow: Lyngbya sp.

3.2. *Quantitative analysis of cyanobacteria by quantitative polymerase chain reaction (qPCR)*

A molecular biology investigation was performed using aliquots of genomic DNA (gDNA), extracted as described in Materials and Methods and utilized as templates in the qPCR, in order to evaluate the relative amount of cyanobacteria present in the biofilm fragments. Using specific *ad hoc* primers designed for this study on the *Cyanobacterium stanieri* gDNA sequence, the target (TrxR) or housekeeping (16S-rRNA) genes were quantified by qPCR experiments. As a control sample (CTRL), gDNA was extracted from the cyanobacteria *Phormidium* sp.*,* collected from freshwater aquaria (Figure 3A and B), and analysed in bright, red and green fluorescence fields. The histogram in Figure 3B shows the result of the qPCR experiments, in which the amount of the TrxR DNA molecules in the fountain biofilm (*) and control (CTRL) are defined. Results highlight that the presence of TrxR DNA molecules found in the control (*Phormidium* sp.) are five times more abundant than in the biofilm samples. This is related to the DNA target molecules available; in fact, starting from an equal amount of gDNA (20 ng), in the control sample only cyanobacteria target sequences are present, instead in the fountain biofilm sample gDNA molecules are available not only from cyanobacteria but also from other organisms.

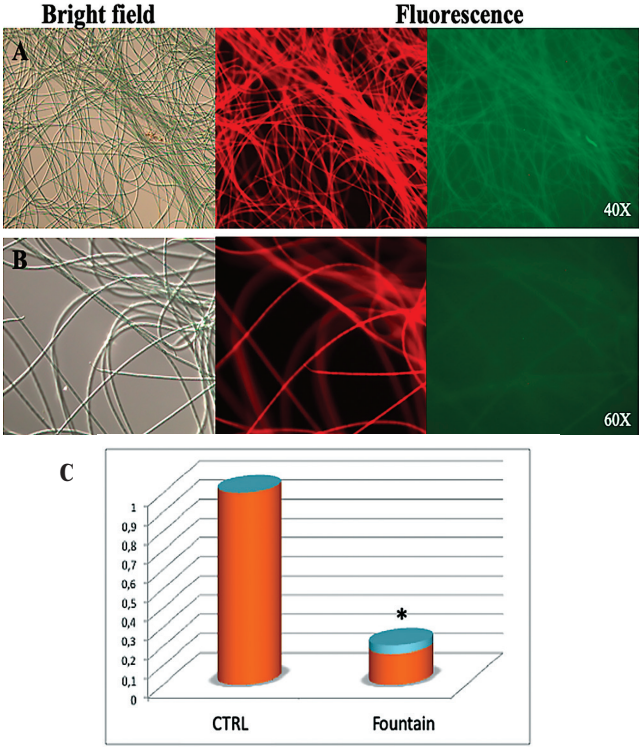

*Figure 3. Morphological profile of Phormidium sp. colonies, observed in bright and fluorescence (red and green) fields; A) = 40X; and B) 60X magnification; C) the result of the qPCR experiments is reported in the histogram: CRTL= amplification product using Phormidium sp. gDNA as template; Fountain= amplification product using fountain biofilm gDNA as template. * indicates a significant difference in amount of DNA target sequence, amount was set equal to 1 on an arbitrary scale of values (y-axis shows Fold Change values).*

3.3. Phylogenetic analysis of TrxR proteins

In order to select an efficient and specific cyanobacteria target gene to perform qPCR reactions, a phylogenetic tree was defined (Figure 4). Specifically, the TrxR protein sequence from six different cyanobacteria were aligned with related sequences of *Homo sapiens* (vertebrate), *Arabidopsis thaliana* (plant), *Chlorella vulgaris* (green alga), *Bacillus* sp. (bacteria) and *Drosophila melanogaster* (invertebrate) by Clustal-O alignment program (Figure 5). All the organisms are summarized in Table 1 with their accession numbers. The phylogenetic tree shows that all cyanobacteria *TrxR* proteins are in the same clade (red square). Instead, *H. sapiens* and *D. melanogaster, A. thaliana, C. vulgaris* and *Bacillus* sp. proteins are separated from cyanobacteria. This result indicates a great difference between cyanobacteria and animals, plants or bacteria protein sequences, confirming the specific qPCR amplification of cyanobacteria target sequences.

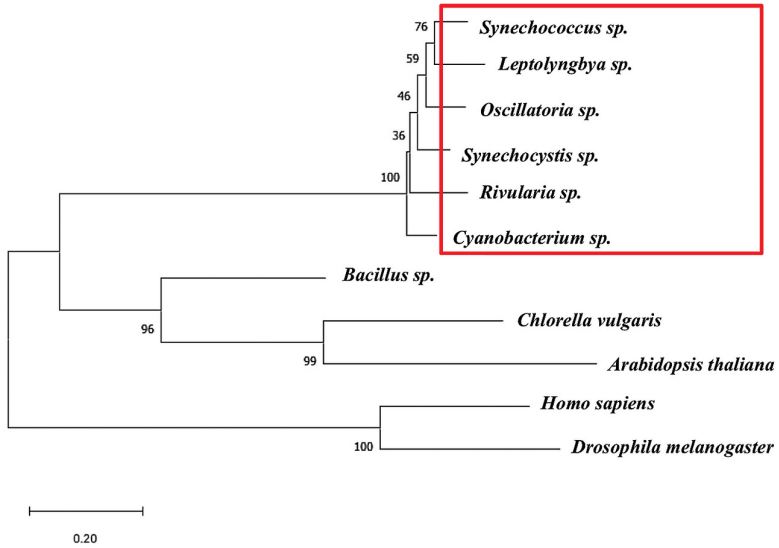

Figure 4. Phylogenetic analysis of TrxR proteins from different organisms. The percentage of replicate trees (1000 replicates) are shown next to the branches. The red square indicates all cyanobacteria species.

Alignment of TrxR amino acid sequences (Figure 5) confirms the specificity of amplification performed by primers designed in this study. Specifically, amino acid sequences corresponding to *Cyanobacterium* sp. are highlighted in pink, while yellow indicates the amino acid sequences of other cyanobacteria (*Rivularia, Oscillatoria, Geminocystis, Synechococcus, Leptolyngbya*); clear differences are detectable for the other organisms analysed (*Homo, Bacillus, Chlorella, Arabidopsis*). Moreover, the percentage of similarity among the TrxR proteins is reported in Table 1, showing a high percentage of similarity among cyanobacteria (96-97%) and a low percentage of similarity in other organisms, bacteria, animals and plant sequences (ranging from 53 to 58%).

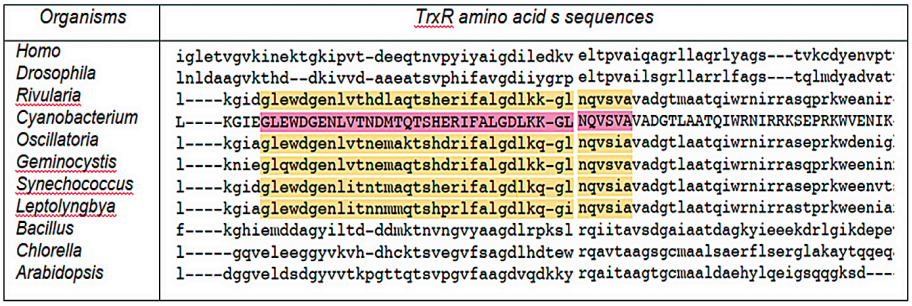

Figure 5. Clustal-O Multiple Alignment of TrxR protein portions from different organisms: pink= amino acid sequence specific for Cyanobacterium sp., amplified by qPCR; yellow=amino acid sequences of other Cyanobacteria genera (percentage of similarity = 96-97%). The other organisms show an evident difference in amino acid sequences of TrxR protein (percentage of similarity = 53-58%).

4. Discussion

The present study provides supplementary information to a previous investigation carried out by Di Carlo et al [23] on cyanobacteria-rich biofilms colonizing the surfaces of an ancient marble fountain (dating back to 1630) located in the city centre of Palermo, Sicily, Italy. In Sicily, winters are mild and summers are very hot which, together with the high levels of humidity on the fountain's surfaces, strongly influence microbial growth giving rise to remarkably complex biofilms. A biofilm or biological patina represents a widespread phenomena in nature and is a three-dimensional structure in general established by a consortium of microorganisms in which cells stick to each other [24]. In the biofilm, microorganisms are fully surrounded by an extracellular matrix composed of polysaccharides, proteins, lipids and DNA. This mucilaginous material is a fundamental feature for the protection and survival of the organisms, allowing them to adhere to the substrate [14]. Environmental conditions determine the degree of colonization, the type of community, the biofilm composition and structure.

The marble of the fountain is a rock of calcium carbonate, with porosity, roughness, and water absorption, which acts as an appropriate support for the growth of microorganisms due to the source of readily available water [25]. Environmental parameters, like humidity, pollution and temperature, also influence the colonization process of the microorganisms [26]. Consequently, as an example, significant differences between the cyanobacterial populations in polluted cities compared to rural unpolluted sites were observed. Different habitats or polluted environments can cause lower biodiversity, thus promoting the growth of only the more resistant taxa, as reported for the genus *Scytonemin* [27].

Cyanobacteria are present in a wide variety of terrestrial habitats (including rocks and deserts) as well as in modern and ancient buildings [28]. It is well known that cyanobacteria induce deterioration of stone cultural heritage (monuments and stone artworks) and, in European countries of the Mediterranean Basin, about 172 taxa have been described, including 37 cyanobacteria (blue algae) and 48 chlorophyta (green algae) genera. The most widespread and commonly reported were *Gloeocapsa, Phormidium* and *Chroococcus* as cyanobacteria taxa [29-30].

The cyanobacteria found in our case study are the most widespread in the Mediterranean basin, e.g. *Gloeocapsa, Chroococcus, Oscillatoria, Lyngbya* and *Microcoleus*, which have a gelatinous sheath that is sometimes pigmented and acts as a reservoir of water, and play an important role in adhesion to the substratum. The deteriorating action of microorganisms on works of art have received substantial consideration in the last few years. Knowledge of the microbial populations living on works of art is essential in order to monitor and keep any deterioration process under control, as well as programme appropriate consequential conservation treatments [29]. Moreover, Chroococcales, Oscillatoriales and Nostocales, are also targeted as toxic cyanobacteria, since they produce toxins that can be detrimental for human health [31]. Therefore, early identification of cyanobacteria colonies allows an adequate strategy to be set up, which can act on microbial vitality and provide for the removal of undesired biofilm.

However, cyanobacteria are characterized by notable morphological, physiological and genetic diversity, consequently methods for identifying cyanobacteria need to be specifically calibrated. Morphological profiles on cyanobacteria performed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) have been reported by Marinè 2004 [32] and Ranalli 2005 [33], but in very few studies has fluorescence microscopy been applied [34]. In addition, different methods have been applied for microscopy observation, such as those methods that use the fixing of microbial cells with formalin [2] or staining them with Lugol's Iodine reactive [35].

Indeed, the direct analysis of secreted cyanotoxins by analytical methods, such as liquid chromatography/mass spectrometry (LC/MS) and immunoassays such as ELISA (Enzyme Linked Immunosorbent Assay) [36], has been implemented with respect to monitoring strategies. These methods are sensitive enough to reveal cyanobacteria toxins but can have limited application and represent only a first level for cyanobacteria identification. However, by combining different investigation methods, exhaustive results can be obtained [37].

Here, we exploited the known autofluorescence of cyanobacterial cells [34]. We used an optical microscope with fluorescence to unequivocally detect the genus of cyanobacteria present in the biofilm taken from the marble fountain of the Two Dragons in Palermo. Based on the presence of photosynthetic pigments (chlorophylls and phycobiliproteins), cyanobacteria were revealed in the biofilms on the marble fountain, using specific fluorescence filters [9,34].

Furthermore, cyanobacteria are very heterogeneous also from a genetic point of view. In spite of a general genetic divergence between genera, there is considerable intragenus diversity and there is no unanimous consensus on which molecular markers are eligible to reach an unambiguous identification.

Therefore, another approach to detect the presence of cyanobacteria was to conduct a molecular investigation using DNA-related assays, mainly based on PCR reaction tests followed by restriction enzyme analysis or DNA sequencing. The 16S-rRNA (16S-ribosomal RNA) molecular locus is an effective marker for the molecular identification of cyanobacteria at the genus level, as well as ITS (Internal Transcribed Sequence of rRNA) [38].

In the last decade, qPCR has been used to quantify cyanobacteria populations present in biofilms, including toxic ones [39]. Indeed, the quantitative estimation of bacterial cells within an environmental sample by real-time quantitative PCR (qPCR) represents an excellent strategy to reveal the potentially toxic cyanobacteria popula-

tion [40]. Al-Tebrineh and co-workers, applied the qPCR method for the detection and quantification of hepato-toxigenic cyanobacteria in bloom samples, using *mcyE* and *ndaF* gene primers, indicating that this technology does effectively offer advantages over traditional protocols used to assess water quality [41]. Although many target/reference genes were selected to identify cyanobacteria species [42], in this study, qPCR was performed using TrxR as the target gene and 16S-rRNA as the housekeeping gene, in order to quantify cyanobacteria *in toto*. The TrxR is a homodimeric 70-kDa flavoprotein in bacteria, fungi and plants with redox active enzymes able to reduce thioredoxin (Trx). Two classes of TrxR were previously identified, one in bacteria and one in eukaryotes. In the higher eukaryotes, TrxR are of about 120 kDa, with a wide substrate specificity that also reduces non-disulphide substrates such as vitamin C [43] thioredoxin reductase and NADPH, the thioredoxin system, is ubiquitous from Archea to man. Thioredoxins, with a dithiol/disulfide active site (CGPC. As a control sample we used the cyanobacteria *Phormidium* sp. collected in a freshwater aquarium. Compared to the control, a decrease in the quantity of the TrxR gene was found in the fountain biofilm, indicating a reduced quantity of cyanobacteria (due to the content of other organisms in addition to the cyanobacteria) with respect to the control sample containing only cyanobacteria.

Phylogenetic analysis demonstrated that the qPCR amplified product was specific for cyanobacteria and not for other contaminant species, as the TrxR proteins of the cyanobacteria clustered separately from other species such as bacteria, invertebrates, vertebrate animals and plants. The use of the TrxR gene in qPCR experiments does not discriminate the single species of cyanobacteria but determines the specific quantization of cyanobacteria when compared with other taxa found in the microbial consortium.

5. Conclusion

In this study we have attempted to define easy but efficacious methods to detect cyanobacteria in biofilms collected from different sites, as well as stonework surfaces, based on fluorescence microscopy and molecular biology (qPCR) investigation. Moreover, we propose the use of the TrxR gene as an efficient molecular marker for quantitative analysis.

It is known that cyanobacteria are the most important colonizers of stonework surfaces in outdoor environments, as they are able to survive changes in climatic and environmental conditions. They are responsible for biodeterioration, but can create a biofilm which encourages the growth of other deteriogens such as fungi or lichens [27], as well as representing a potential danger to human health [39-40].

Therefore, early diagnosis, also using biotechnological protocols, allows adequate prevention strategies to be defined in safe conditions, in addition to taking action before irreversible surface changes occur.

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Summary

Cyanobacteria or blue algae are ubiquitously present in both fresh and brackish water environments. They also grow in conditions of high humidity, colonizing stones or monuments and fountain surfaces, and creating thick biofilms able to induce biodeterioration in the constituent materials of artefacts. As well as several photoautotrophic organisms, cyanobacteria belong to the microorganisms identified as primary colonizers, playing an important role in stone artwork deterioration.

In this study, an analysis was made of the biofilm collected from the stone fountain of the Two Dragons in Palermo (Italy), revealing the presence of cyanobacterial colonies by optical microscopy, due to their peculiar auto-fluorescence. Furthermore, molecular investigations by qPCR (quantitative Polymerase Chain Reaction) were utilized to gather quantitative information, and phylogenetics analysis was used to confirm the *Thioredoxin reductase* (TrxR) gene as a suitable molecular marker. The results highlight the presence of cyanobacteria as the main taxa, whose growth is induced by microclimatic and environmental conditions, and by the physical characteristics of the stone surface.

Identification of microbial populations living on stone artworks is the starting point for successful control and conservation strategies, which can help to define the correct protocols to block cellular activity and to find appropriate methods for removing biofilm, as well as counteracting possible recolonization.

Riassunto

I cianobatteri o alghe azzurre sono ubiquitariamente presenti in ambienti sia di acqua dolce sia salmastra, sviluppandosi anche in condizioni di elevata umidità, colonizzando pietre o superfici di monumenti e fontane, e generando complessi biofilm, in grado di indurre il biodeterioramento dei materiali costitutivi del manufatto. Così come per al-

tri organismi fotoautotrofi, i cianobatteri appartengono ai microrganismi identificati come colonizzatori primari, svolgendo un ruolo importante nella formazione di consorzi microbici in grado di indurre processi di deterioramento.

In questo studio si è analizzato il biofilm presente sulla superficie di una fontana in materiale lapideo, rivelando, mediante microscopia ottica sia in campo chiaro sia in fluorescenza, la presenza di colonie di cianobatteri con peculiari morfologie e strutture. Inoltre, la tecnica qPCR (quantitative Polymerase Chain Reaction) ha permesso di confermare il gene della *Tioredossina reduttasi* (TrxR) come marcatore molecolare adatto per studi di filogenesi. I risultati evidenziano la presenza di cianobatteri come principali taxa, la cui crescita è indotta dal microclima e dalle condizioni ambientali, oltre che dal litotipo e dalle caratteristiche fisiche della superficie lapidea.

La più completa identificazione delle popolazioni microbiche che colonizzano manufatti storico-artistici è peculiare per la definizione di un'adeguata strategia per il controllo delle attività microbiche, per la rimozione del biofilm, oltre al contrasto di eventuali ricolonizzazioni.