



# Investigation on Microbial Biodeterioration of the Stone Monuments in Yildiz Technical University—Yildiz Campus—Istanbul—Turkey

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## Abstract

The stone samples of historical monuments around Yıldız Technical University Besiktas Campus were investigated using DNA extraction-PCR-DGGE methods, scanning electron microscopy (SEM), XRF, and other analytical methods to assess stone decay over the centuries. Microbial diversity was examined by classical cultivation and modern diagnostic methods besides modern analysis techniques. The number of the microorganisms in per gram of stone samples was calculated by microbial culture methods. SEM analysis showed that stone surfaces have too many pores, decaying pieces and microbial colony. It is put forth by XRF analysis that stone materials have some elements serving the growth of microorganisms. It was concluded that there is a close connection the stone structure and microbial growth, most likely mineralogical composition, hardness and porosity of stone. Cyanobacterial microorganisms lived on stone surfaces were also determined using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. It was revealed DNA-based molecular analysis of 16S rRNA that 23 bacterial/Cyanobacterial clones were inhabited to stone materials.

## Introduction

Istanbul has been protecting its characteristic of being a historical, architectural, cultural and business center for years and has numerous historical buildings which reflect its rich and long-lasting history [1]. The historical peninsula has a strategic geographical location bounded by “The Bosphorus” and connecting the Aegean and Black Sea. In addition, because of the most strategical, historical and cultural city in the world, as well as Europe and Asia, it has been declared the European Capital of Culture for 2010 by the European Union [1, 2].

Besides these unique beauties of Istanbul, it has thousands of historical buildings that have been decaying for many years exposed to open air conditions. Decaying

processes or biological decomposition of natural waste materials is often the most effective way of waste disposal. For this reason, biodegradation or biodeterioration has mentioned in undesirable natural events. The basic structural elements of the historical monuments are stones, and it has open air conditions throughout the history with meteorological factors. The alteration and decaying processes of stone is basically formed by natural and anthropogenic impacts influencing various physical, chemical and biological damage factors. At the climax conditions the microbial biofilms occur on stone surfaces and it protects against a variety of environmental stresses, such as changing sunlight radiation, pH, osmotic stress, dehydration and to provide a habitat for microorganisms [3–5].

The biodeterioration of stone monuments is a complex process, which is caused by the interaction of many physical, chemical and biological agents contribute to the deterioration of historical buildings. Degradation of stone materials under permanently open air conditions is leading to the destruction and the propagation of microcracks [3–6]. Biodeterioration processes also affect many living creatures such as bacteria, microalgae, cyanobacteria, bryophytes, mosses, fungi, insects, rodents, birds, herb and human. The microbial organisms can cause staining, cracking, powdering, disfigurement and displacement of building material, which leads to the permanent loss

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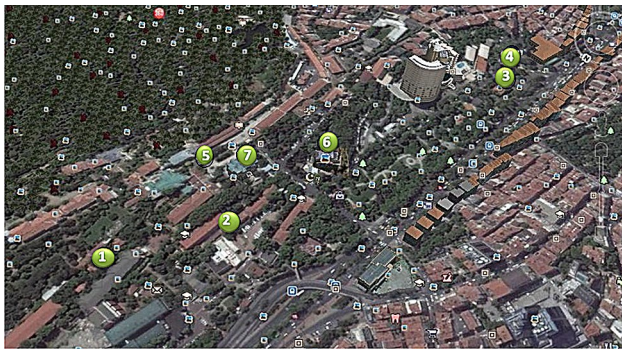
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of stone monuments besides aesthetic damage and colorizations. Microalgae and cyanobacteria are usually the precursor deteriorating agents of stone buildings due to their photosynthetic nature. These microorganisms can deteriorate stone either chemically or mechanically and their presence is generally detected through the formation of patina or crusts [3–5].

The damage of the microorganisms on the stones on historical buildings based on scientific evidence has been carried out since 1930s. Researches have been made on the decay of historical monuments. Some of these studies have been cited as references in our research [3–6].

The aim of this study was to determine to biodeterioration of stone monuments in the specific area of the world's great metropolises which are containing important works of the ottoman empire, around of the Yildiz Technical University—Besiktas Campus.



**Fig. 1** The research areas

## Material and Methods

### Sampling Sites and Sampling Methods

The microbial analyzes carried out in February, May, August and November 2017 and 2018, and the number of microorganisms, the species and colony configuration pattern on stone surface were determined by DNA extraction-PCR-DGGE, scanning electron microscopy (SEM), XRF, optical microscopy and classical microbial culture methods. The stone samples from the outer surface of 0.5 and 2-m-high parts of seven historical buildings in around of Yildiz Technical University—Besiktas Campus were taken by means of sterile plastic bags. The research areas are shown in Fig. 1.

Microbiological studies were carried out in sterile conditions. The original construction methods and materials have been investigated in a comprehensive manner, and all of these historic buildings have been restored in accordance with the original form. Therefore, it couldn't always get samples from all sampling points of monuments. Sampling points, sampling heights and stone types are given in Table 1.

### Optical Microscopy, SEM and XRF Examinations

The samples were broken into small pieces under aseptic conditions. Optical microscopy studies were carried out with OLYMPUS BX51 model polarizing microscope extended wavelength range between UV and IR with 5 megapixel high-precision digital camera combined with computer control system. For the optical microscopy examinations, the preparations were stained with methylene blue, malachite green and epoxy resin, and were observed under the microscope [4, 5, 7]. SEM and XRF analyzes were carried out

**Table 1** The characteristics of the sampling points

Sampling points	Height	Symbol	Stone type
Yildiz Palace—Prince mansion wall	0.5 m	YP- PMW1	Volcanic (basalt)
	2.0 m	YP- PMW2	
Yildiz Palace- The Hollow Palace	0.5 m	YP- THP1	Volcanic (basalt)
	2.0 m	YP-THP2	
Ertuğrul Tekke Mosque	0.5 m	ETM1	Limestone (M.S.Stone)
	2.0 m	ETM2	
The Seyh Muhammed Zafiri tomb	0.5 m	ŞMZT1	Limestone (M.S.Stone)
	2.0 m	ŞMZT2	
Yildiz Palace- Hedge Pavillion	0.5 m	YP- HP1	Limestone (M.S.Stone)
	2.0 m	YP- HP2	
Yildiz Hamidiye Mosque	0.5 m	YHM1	Limestone (M.S.Stone)
	2.0 m	THM2	
Yildiz Palace-Great Mabeyn Pavilion	0.5 m	YP- GMP1	Limestone (M.S.Stone)
	2.0 m	YP- GMP2	

at the Cekmece Nuclear Research and Training Center and Istanbul Technical University (ITU). MEM-TEK National Research Center on Membrane Technologies. XRF analyzes were performed on BRUKER S8 TIGER model energy-dispersive X-Ray Fluorescence Spectrometry (WDXRF) with the Axios Advanced wavelength controlled by the Super Q software program. The elemental composition of stone samples was investigated under a 1-mm SR beam. The operating conditions were 40 kV and 0.5 mA. The settle-way of the microorganisms on the surface of the stones was determined by different microbiological and surface analytical techniques complemented by SEM–EDS combined system energy-dispersive spectrometer. SEM analyzes were performed on FEI—Quanta FEG 250 MODEL environmental scanning electron microscopes. The samples are covered with Gold palladium alloys in the nanometer degree, and thus the samples were ready to be examined as natural form in SEM. After the samples coated in a nanometer degree were attached in the holder of SEM, they were placed into the vacuum chamber to photograph. Secondary electron images of surfaces of the stone samples were obtained at 3–30 keV energy level,  $\times 100$ –20,000 magnifications [4, 7].

### Determining the Total Cyanobacteria and Fungi

Samples were taken from the parts of 0.5 and 2 m high from the ground of the monuments and the incubated colony forming unit (CFU) in 1 g of these samples were found out in similar studies [8]. The number of the microorganisms in one gram of stone samples was calculated by microbial culture methods. One gram of crumbled stone samples was put into 250-ml flasks containing physiological water (9 g NaCl in 1 L double distilled water) and mixed at 120 rpm for 30 min in. The microorganisms in/on the stone samples were transferred to the water medium. The following incubation studies were done from this liquid medium. 1 ml of samples taken from these liquid media are incubated on sabouraud dextrose agar (SDA), Malt extract agar (MAE), dichloran chloramphenicol peptone agar (DCPA) and Potato Dextrose Agar (PDA) for incubating of mold and fungi. Streptomycin (50 mg/L) was used to prevent the development of cyanobacteria in conventional cultivation methods carried out with agar medium. During the counting of bacteria, nystatin (50 mg/L) was also added in SPCA and DCA [8].

The microbial pre-cultivation studies were carried out at different temperatures ranged between 5 and 40 °C, and the optimum incubating temperatures of the microorganism groups were found out. The optimum incubating temperature of the microorganisms was determined as 20 °C for bacteria and fungi for these microecological conditions. The cultures were incubated at this temperature and examined every day for two weeks. After that, the isolates were passed through tubes into petri dishes containing SDA and

PDA for determination of fungi isolates. The isolates were examined macroscopically following staining with lactophenol cotton blue. The identification of fungi was based on their macroscopic characteristics as a result of germ tube tests and biochemical tests. For identification of the fungi, a small portion of each sample mounted in a few drops of 20% potassium hydroxide was examined for the presence of characteristic fungal elements and diagnostic morphology [4, 6, 9].

### The Identification of the Bacterial and Cyanobacterial Strain Via Advanced Methods

The microbial diversity was profiled by DNA extraction and the PCR-DGGE of partial 16S rRNA genes followed by their sequencing [10]. The extraction of DNA from the samples was performed using a Power Soil DNA isolation kit (MO BIO Laboratories, Inc., USA) using the manufacturer's instructions. DNA was extracted using the NucleoSpin® DNA Isolation Kit (Macherey–Nagel, Duren, Germany). Bacteria and cyanobacteria species were determined by applying PCR and DGGE-based molecular techniques to 16S ribosomal RNA genes after DNA extraction. Diagnosis of microbial species consists of the following stages; DNA extraction, PCR (Polymerase Chain Reaction), DGGE (Denaturation Gradient Gel Electrophoresis) and Nucleic Acid Sequence analysis. Stone samples taken from research points were prepared for use in molecular techniques and nucleic acids of bacterial species in samples were extracted using the NucleoSpin® DNA Isolation Kit and stored at -20 °C until the next analysis stage. The 16S rRNA genes in the nucleic acids extracted using the PCR method were amplified by Thermal Cycler using forward and reverse primers 357F-GC and 907R primers. In order to demonstrate the diversity of microbial species, the DGGE study was performed to determine the Nucleic Acid Sequence. The following devices were used in this molecular-based study; PCR (BIO-RAD T100™ Thermal Cycler System), Electrophoresis Device (SCIE-PLUS), DGGE (BIO-RAD D-Code™ Universal Mutation Detection System) and Gel Imaging System (WiseDoc). The resulting 16S ribosomal RNA nucleotide sequences of the resulting bands were copied in the form of A-G-C-T index files and evaluated on the BLAST program on the website <https://blast.ncbi.nlm.nih.gov/> and probable microorganism types were identified. In DNA isolation, samples from research sites were purified according to the NucleoSpin® DNA Isolation Kit procedure. Species determinations were made following the NucleoSpin® working procedure. The isolated DNAs were stored at -20 °C until PCR. 16S rRNA genes were amplified by PCR amplification using the BIO-RAD Thermal cycler. Primers and nucleotide sequences are shown in Table 2.

**Table 2** Primers and nucleotide sequences used in the research

Primer Name	Nucleotide sequence
357F-GC	5'-CGCCCGCCGCGCGGGCGGGGCGGGGCG GGGGCACGGGGGG CCTAC GGGAGGCAG CAG-3'
907R	5' CCGTCAATTCMTTTRAGTTT-3'

Enzymes and primers were maintained at 4 °C during the procedure and enzymes were added to the reaction at the latest stage. Obtained nucleotide sequences were evaluated in the “BLAST” diagnostic program on the internet site <https://www.ncbi.nlm.nih.gov/> and species identification was performed by reporting the similarities of the existing species identified in the database according to possible differences.

Sequences of all samples obtained after sequence analysis are listed using “CHROMAS 2.6.2, UNIPRO UGENE” software. 16S rRNA broad base sequences were obtained and typed by BLAST analysis. The ranked sequences were compared with the reference sequences in the “BLAST” program and the access numbers (Table 3) recorded in the GenBank database were determined. Phylogenetic analyzes were carried out using the “neighbor joining” method and a phylogenetic tree was created using the “UNIPRO UGENE” software.

## Results and Discussion

### Determining of Mineralogical Composition of Stones and Its Effects on Microorganism Growth

In this research, via XRF analysis, Ca, K, Fe, Ti, Sr, Ni, Ba, Mn, Sn, Pb, W, Zr, Cu, Rb, Se, S, Bi, As, Co, Cl, Zn, V, Cr, P elements are found in stones of historical monuments (Table 4). In the analysis of the Fence Pavilion, the ratio of Sn (Tin) was found to be high compared to other samples. The presence of Sn indicates that there is corrosion activity in the environment where the sample is taken [4, 9, 11]. Sn element was not found in samples taken from the 2.0 m height of Ertugrul Tekke Mosque, Sehzade Pavilion, Sheikh Mohammed Zafiri Tomb and 0.5 m high of Cukur palace and Sehzade Pavilion.

Calcium chloride, sodium chloride, potassium chloride, such as substances located in the rock with the effect of water dissolves over time, causing the formation of gaps in the rock. Furthermore, when calcium sulfate which enters into porous and porous rocks and fills these voids, reacts with water, it presses the walls of the swelling cavities. As a result of this, thin capillary cracks are first seen and then they are enlarged and ruptured from the rocks [3, 4, 9, 12]. This means there is an active decomposition in the presence

of chlorine, potassium and calcium in stone samples 1 and 3, and passive decomposition in the absence of chlorine. The Pb value of Sehzade Pavlion is higher than the other stones which it makes the material soft and flexible [10] (Table 4).

Chemolithotroph microorganisms oxidize the inorganic substances in the stone and carry out their metabolism with the energy they obtain. Minerals such as iron and manganese found in metals in solid minerals serve as microorganisms for electron respiration. This mechanism supports biogeochemical circulations such as nitrogen, sulfur and carbon cycle in the anoxic zone on the stone surface. Fungi also play a key role in the biogeochemical conversion of nutrients and metals that are necessary for the growth of living organisms in the biosphere [13, 14].

In addition, the lime compound adhesive mortar used in historical monuments is the ideal reproduction area for microorganisms. Chemotrophic bacteria, which use hydrogen and carbon as energy sources, produce complex corrosive organic acids and oxidize iron and manganese minerals. Bacteria play a role in the formation of calcium nitrates in limestones [3].

### SEM Study and Effect on the Growth of Microorganisms

SEM and XRF studies were performed to determine the hardness, porosity and mineralogical structure of the stone. Some of the images obtained from SEM studies are presented Fig. 2a–d. These studies have provided to visualize the determination of degradation on the stone material and the structural relations of the degradation via microorganisms (Fig. 2a). It is seen that the rocks in the Fence Pavilion and Ertugrul Tekke Mosque are porous and create an environment suitable for the settlement of microorganisms.

Figure 2b shows the hyphae and sporangia of *Penicillium* sp. and *Micromyces* are commonly found on stone surface. Especially *Basidiospor*, *Askospor* and *Penicillium* spores are very diffuse. It is seen that the Fig. 2b, c, the spores and micro fungi formed into stone pores and surfaces. When the SEM images are examined, the microbial biofilm is clearly seen in Fig. 2d images.

The effects of microorganisms living on the stone surface are based on two opposing theories. The first, microbial biofilm formed on the stone surface dissolves minerals by the enzymatic-chemical mechanism to break down the stone. The second theory about this layer; as it cuts the contact of the stone with the external environment, it prevents the fragmentation of the stone [9, 15].

In Fig. 2a, the degradation on the stone surfaces can be clearly seen. In sample 2b, *Penicillium* sp. hyphae, microbial biofilms, ascospores and basidiospores are seen. The most important feature of the Fig. 2 d is the predominance of microbial biofilms formed on the stone surface. Biofilms

**Table 3** The sample points, bacterial and cyanobacterial strains and Genbank access numbers used to the phylogenetic tree

Bands	Sample points	Blast references of strains		Gen Bank Access Numbers	16S rRNA Similarity (%)
		The closest relative of strain	Strain		
1	YP- HP1	<i>Calochaete cimrmanii</i>	RKST551	NR_117712.2	94
2	YP- HP1	<i>Cylindrospermum pellucidum</i>	CCALA 989	NR_125683.1	93
3	YP- HP1	<i>Cylindrospermum licheniforme</i>	CCALA 995	NR_125686.1	93
4	YP- HP1	<i>Microcoleus</i> sp. PCC 7113	PCC 7113	NR_102467.1	92
5	YP- HP1	<i>Calothrix</i> sp. PCC 7507	PCC 7507	NR_102891.1	93
6	YP- HP1	<i>Gloeocapsa</i> sp. PCC 7428	PCC 7428	NR_102460.1	93
7	YP- HP1	<i>Scytonema hofmanni</i>	PCC 7110	NR_112180.1	89
8	YP- HP1	<i>Arthrospira platensis</i>	PCC 7345	NR_125711.1	
9	YP- HP1	<i>Lyngbya aestuarii</i>	PCC 7419	NR_114680.1	91
14	YP- HP1	<i>Chitinophaga jiangningensis</i>	JN53	NR_118590.1	90
15	YP- HP1	<i>Chroococciopsis thermalis</i>	PCC 7203	NR_102464.1	87
16	YP- HP1	<i>Chlorogloeopsis fritschii</i>	PCC 6912	NR_112176.1	90
17	YP- HP1	<i>Hassallia antarctica</i>	CCALA 957	NR_117192.1	83
18	YP- HP1	<i>Nostoc</i> sp. PCC 7120	PCC 7120	NR_074310.1	82
19	YP- HP1	<i>Brasilonema terrestre</i>	CENA116	NR_116034.1	94
20	YP- HP1, YP- PMW1, YP- THP1, ETM1, ŞMZT1, YHM1, YP- PMW2, YP-THP2	<i>Oscillatoria nigro-viridis</i>	PCC 7112	NR_102469.1	91
21	YP- HP1, YP- PMW1 YP- THP1, ETM1 ŞMZT1, YHM1 YP- THP2, YP- PMW2, YP- THP2	<i>Nostoc punctiforme</i>	PCC 73,102	NR_074317.1	89
22	YP-HP1	<i>Halospirulina tapeticola</i>	CCC Baja-95 Cl.2	NR_026510.1	93
23	YP-HP1, YP-PMW1, YP-THP1 ETM1 ŞMZT1, YHM1 YP-PMW1 YP-HP2	<i>Anabaena cylindrica</i>	PCC 7122	NR_102457.1	91
24	YP-HP1	<i>Fischerella thermalis</i>	PCC 7521	NR_112178.1	94
25	YP-PMW1 YP-THP1 ETM1, ŞMZT1, YHC1	<i>Bacillus thermoamylovorans</i>	LMG 18,084	NR_117028.1	91
26	YP-HP1	<i>Truepera radiovictrix</i>	RQ-24	NR_074381.1	86
27	YP-HP1	<i>Acidicaldus organivorans</i>	Y008	NR_042752.1	83

**Table 4** XRF analysis results of the historical monuments

Sample no	Ca	K	Fe	Ti	Sr	Ni	Ba	Mn	Sn	Pb	W	Zr	Cu	Rb	Se	S	Bi	As	Co	Cl	Zn	Cr	P
1	> 10%	9984	5692	1068	183	-	113	167	-	19.9	-	32.6	-	22.5	-	5.6%	15	6.5	5.1	549	28	-	-
3	> 10%	7173	8172	1459	123	-	103	234	-	95	-	31.9	-	10.3	-	5.01%	14	9.9	9.6	4690	61	-	-
4	> 10%	1,45%	1.94%	3227	263	-	303	450	-	39.4	-	70	51	40.5	3.0	> 10%	33	10.1	19.7	-	56	186	8826
6	> 10%	9358	1.6%	3675	325	-	199	168	-	55	-	108	26	54.1	-	> 10%	-	5.8	18.4	-	92	200	-
8	> 10%	1,55%	3009	485	319	-	319	88	-	69	-	36	12	15.7	-	3.69%	-	9.1	-	-	361	177	-
9	> 10%	1,68%	4583	704	380	102	380	86	51	30	20	20	20	12.8	3.6	-	-	-	-	-	-	-	-

Since the sensitivity of the device decreases and when it rises above 10,000 ppm, it gives the analyzed results as a percentage (%)

have established microorganisms, especially bacteria. Figure 2 b, c and d, gives the appearance of *Penicillium* sp. hyphae, ascospores and basidiospores. In particular, it is an example of a common lifestyle called “microbial consortia”.

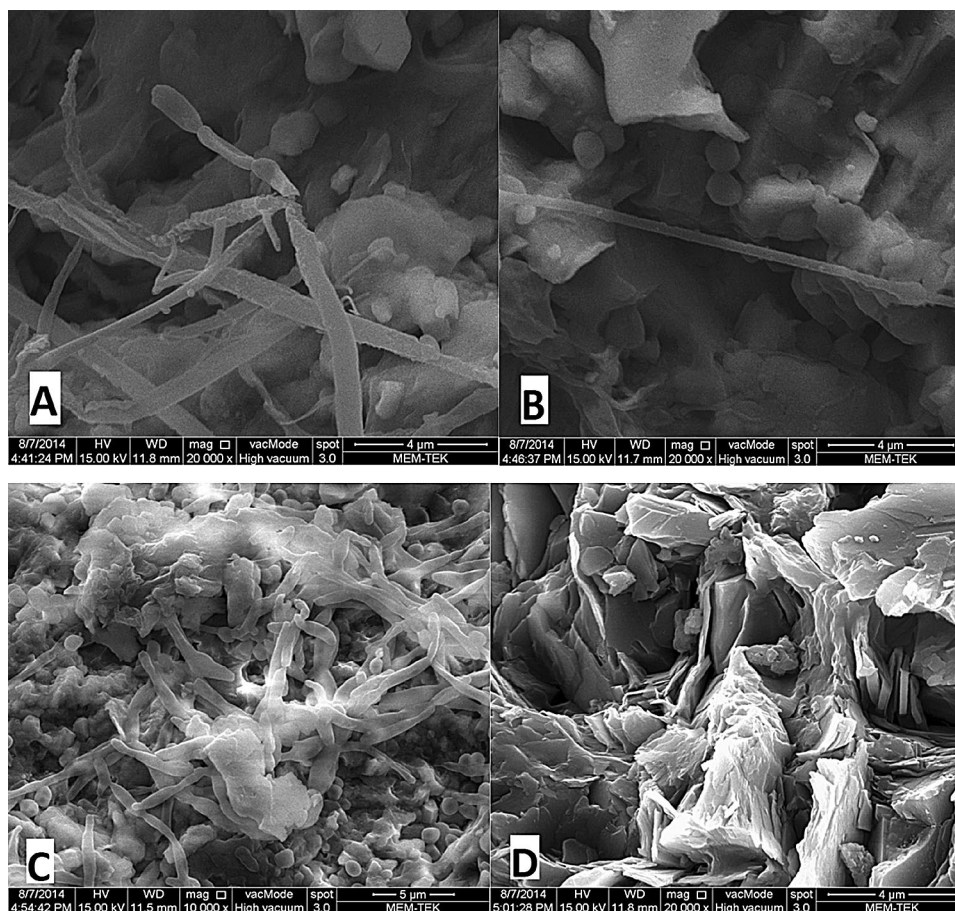
### Diagnostic Examination Studies Based on Classical Media Studies

Although many species of microorganisms cause the degradation of the stones in the historical monuments, bacteria have an important effect [16]. Bacteria show autotrophic and heterotrophic metabolic activity during the decomposition of minerals in stone structure. Since the primers used in PCR analysis adapt to the gene structure of a certain group of bacteria or microorganisms, all bacteria and microorganisms found in the stones cannot be detected by the PCR method. For this reason, classical media analyzes, which have a much wider spectrum, were also used in the diagnosis and counting of bacteria and fungi. In the first stage of the research, microorganisms such as bacteria, cyanobacteria and fungi were found in 1 g of the stone material and the number of the colonies that were capable of reproduction was determined by developing colonies. Subsequently, the species or genus were determined and the number of viable bacteria (CFU) in 1 g stone sample was calculated by classical culture methods. The species components are given in the Table 5.

It is seen that the number of microorganisms is low in June–July when rain is low and it develops much more on stone surface in March and December. This is thought to be due to the difficulty in the ability of water to be retained on the stone surface. When the results obtained from the samples in the previous months are compared with February–March period, it is seen that the number and species of microorganisms differ between the research areas. In June and July period, species diversity among the research areas was high, while the total number of microorganisms was low. Some microorganism species reached the highest level in late summer. However, the number of individuals was found to be low, which is in line with the general rule of life in the ecosystems.

During the study, bacteria species were determined in the samples taken from the Fence Pavilion and the identified bacteria are given in Table 5. These bacteria have been detected in most structures. *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus agri* and *Paenibacillus* sp. and *Staphylococcus* sp. were determined and air pollution was found to be effective on microbiological degradation of stones. When scanning electron microscope photographs were examined, biological degradation of *Bacillus* sp. belonging to the genus is clearly seen on the surface of the stones. *Bacillus* sp., *Paenibacillus* sp., *Staphylococcus* sp. are one of the bacteria that contribute to the biological degradation of stones.

**Fig. 2** SEM analysis of the samples taken from the research points



**Table 5** Bacterial strains living on the stone surface detected by classical cultural method

Microorganisms	Sampling points						
	YP- PMW1 YP- PMW2	YP- THP1 YP- THP2	YP-GMP1 YP-GMP2	YP- THP1 YP- THP2	YHM1 YHM2	ETM1 ETM2	SMZT1 SMZT2
<i>Bacillus subtilis</i>	+	+	+	+	+	+	+
<i>Bacillus licheniformis</i>	+	+	+	+	+	+	-
<i>Bacillus cereus</i>	+	-	+	+	+	-	+
<i>Bacillus agri</i>	-	+	-	+	-	+	-
<i>Paenibacillus</i> sp.	+	+	+	+	-	-	-
<i>Staphylococcus</i> sp.	-	+	-	+	+	-	+

Some of the bacteria found on the stone contribute to the biological preservation of historical monuments. For example, *Bacillus subtilis* significantly reduces the water permeability of the stone so that the stone surfaces are less exposed to the corrosive effect of rain and polluted water. *Bacillus cereus* strains are actively used in biological induction to provide calcite precipitation in strengthening monumental stones [17] and the stone treated as *Bacillus cereus* has been shown to suffer significant water loss. At the same time as a result of bacterial metabolism due to high calcium oxide, especially a stone layer (stone) is formed by *B. cereus* formation on the stone makes it more resistant to physical

stress [8]. Microbial carbonate precipitation (MCP) induced by *Bacillus cereus* significantly reduces the amount of Cr, which decreases the corrosiveness of the stone [18]. Microbial carbonate deposition (MCP) is used for the restoration of calcareous monuments [19].

Chemoheterotrophs are not only found in stone erosion, but also because these microorganism groups have the ability to increase calcium carbonate precipitation, they have effects on the strengthening of rocks [20]. *Bacillus cereus*, one of the chemoheterotrophic bacteria, is involved in the consolidation of plaster and rock by increasing the calcium carbonate precipitation [21].

Heterotrophic bacteria, *Bacillus sp.*, *Paenibacillus sp.*, *Staphylococcus sp.* especially murals, caves and underground cemeteries are common species [21].

*Bacillus subtilis* bacteria produce rough biofilms and it has been found that color changes on stone surfaces due to this biofilms are visible to the naked eye. In addition, bacterial biofilms turn into spore structures and increase calcification. The appearance of *Bacillus subtilis* bacteria on granite stone surfaces starts with dissolution of granite mineral components. In the presence of oxalic acid among its metabolites,  $\text{Ca}^{+2}$  and  $\text{C}_2\text{O}_4^{-2}$  ions are seen in the surface solution and oxalate crystallization begins. In addition, calcium oxalate released by fungal oxalogenesis is metabolized by fungi and oxalotrophic bacteria [16, 17]. Some *Bacillus sp.* species exhibit an effect that accelerates stone degradation by producing surfactants and acids with self-emulsifying activity [3, 17].

## Bacteria and Cyanobacteria Species Analysis with PCR and DGGE Studies

### Phylogenetic Tree Studies

The phylogeny of the genotypes found in GenBank by using sequence analysis results were generated by using phylogenetic tree showing genetic relationships by using UGENE 1.26.1 (Unipro UGENE: Integrated Bioinformatics Tools) program. After sequence analysis, samples from 16S rRNA gene nucleotide base sequences chromatography were determined using the Chromas version 2.6.2 program.

By comparison of DNA sequences, phylogenetic analysis revealed evolutionary kinship relationships between species, genome similarities or differences between organisms. Phylogenetic tree was created by using this information. Phylogenetic analyzes were performed in the Unipro UGENE program according to Neighbor Joining (NJ) model and the following trees (Fig. 3).

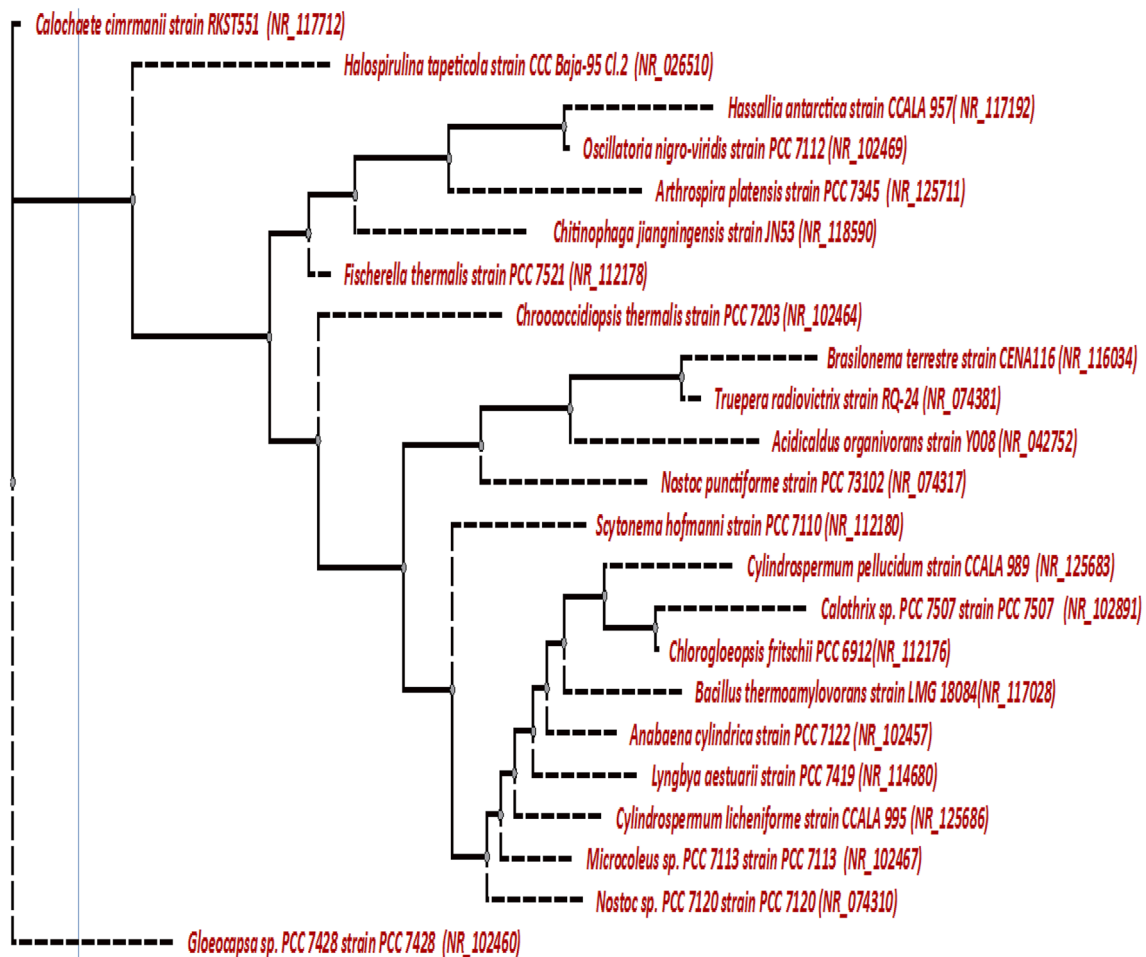


Fig. 3 Neighbor Joining (NJ) model of cyanobacteria / bacterial species obtained by the phylogenetic tree



According to the results of the bacterial strain identification, in the DGGE profile the microorganism species were seen in the samples taken from Sehzade Pavilivion (2 m) and Cukursaray (2 m) exterior wall surfaces. Only 2 bacterial species (bands 21, 23 and 20, 21, respectively) were seen. Bacteria, cyanobacteria and brown pigmented fungi form a living environment suitable for microorganisms by supplying the nutrients needed by microorganisms on the stone surface with the biofilm formed on the stone surface.

In the sample taken from the wall surface 2 m high, Sehzade Pavilion have *Nostoc punctiforme* strain PCC 73,102 (band 21) and *Anabaena cylindrica* strain PCC 7122 (band 23); in Cukursaray, cyanobacteria belonging to genus *Oscillatoria nigro-viridis* strain PCC 7112 (band 20) and *Nostoc punctiforme* strain PCC 73,102 (band 21). *Nostoc punctiforme* is a microbial genus commonly found on stone surfaces receiving relatively high light intensity [21]. This species was found to be dominant in each sample area. *Nostoc punctiforme*, a type of filamentous cyanobacteria, is seen as covering the black-brown biofilm surface on the external surface of the monuments [22]. *Nostoc sp.* genus blue-green algae during the metabolism of mucilage "sticky" substances by producing themselves and other microorganisms to adhere to the surface of the stone and *Oscillatoria sp.* is involved in the biological disintegration of stones together with blue-green algae [3, 23].

*Nostoc sp.* (band 18) and *Gloeo capsula sp.* (Band 6) fixes the nitrogen of the air by converting it to ammonium and nitrates, which provides a living space for other microorganisms and heterotrophic bacteria that will come after the stone surface [24].

Species belonging to bands 20 and 21 were also found in the samples taken from the outer wall surfaces of Ertugrul Tekke Mosque, Seyh Muhammed Zafiri Mausoleum, Yıldız-Hamidiye Mosque and Cit Pavilion. *Nostoc punctiforme* strain PCC 73,102, *Anabaena cylindrica* strain PCC 7122 and *Oscillatoria nigro-viridis* strain PCC 7112 are common in the 20, 21, 23 bands. The band number 25 is different from the other historical building. *Bacillus thermoamylovorans* strain LMG 18,084 was determined as a species in this band. *Bacillus* species on the stone surface form organic acids during their metabolism and these organic acids dissolve elements such as Fe, Mg, Mn, Si, Al, Ca [25]. The building material consisting of limestone, silica and fossil sediments which are known as kufeki stone which is widely used in these structures is *Bacillus sp.* It was also found in the literature that *Bacillus* species are the leading microorganisms that damage calcareous stones [25].

*Cyanobacteria* were found in bands 20, 21, 23. Since cyanobacteria carry photosynthetic pigments of chlorophyll-a, betacarotin, flavianin, phycocysteine, they cause discoloration and physico-chemical degradation in the stones with biofilms during the growth of historical structures [26].

*Anabaena cylindrica* (band 23) is a filamentous and nitrogen-detecting blue-green algae commonly found in freshwater medium. These blue-green algae responsible for nitrogen fixation are species with special thick-walled cells called heterocytes. *Chroococcidiopsis thermalis* strain PCC 7203 detected in band 15 is a unicellular Cyanobacterium that is tolerant to extreme environmental conditions (e.g., extreme heat and cold, dry conditions). *Chroococcidiopsis sp.* and *cryptoendolytic cyanobacteria* live under the rock surface [27].

In the band 1, RKST551 16S strain of *Calochaete cimrmanii* strain belonging to the class of Cyanophyceae, living on the soil surfaces was found. This blue-green algae-hydrated polysaccharides create a sticky mucilage and give out of the cell. This mucilage layer contributes to the formation of colonies containing dozens of microorganism species [28].

In Table 3, *Cylindrospermum pellucidum* strain CCALA 989, which is a filamentous cyanobacteria living in soils, was detected in 93% similarity with the difference of 29 nucleotides. Cyanobacteria can live in rock crevices and crevices and in cavities formed in porous transparent rocks such as sandstone and marble, but not in dense dark volcanic rocks [27].

In the band 4, *Microcoleus sp.* PCC 7113 and PCC 7113 strain was detected. *Microcoleus sp.* (band 4) is a blue-green algae with filament and nitrogen that is commonly found in waters. This blue-green algae, which plays an important role in nitrogen fixation, is highly effective in the development of microbiological colonization, also called "microbial mat". It is also effective on the degradation of stones with photosynthetic pigments it creates. The color changes occurring on the stone surfaces disrupt the aesthetic appearance of the stone. Immediately after wetting these biofilms with rain, *Hassallia sp.*, *Tolypothrix sp.*, *Scytonema sp.*, *Lyngbya sp.* and *Calothrix sp.* type cyanobacteria occur by breeding [21].

In the band 5;, *Calothrix sp.* PCC 7507 and PCC 7507 strain and in band 17;, *Hassallia antarctica* CCALA 957 strain and in band 9; *Lyngbya aestuarii* PCC 7419 strain and in band 7; *Scytonema hofmanni* PCC 7110 strain were detected. Blue-green algae of the genus *Scytonema hofmanni* show development on stone surfaces and grounded shells [29]. The fence pavilion was *Calothrix sp.* (band 5) is a blue-green algae that may have a heterocyte structure that forms a solid black biofilm on the upper surfaces of the stone [30]. No species identification has been found in the literature that matches the *Hassallia Antarctica* strain CCALA 957 (band 17). This microbial strain belongs only to this region.

The *Nostoc sp.*, *Calothrix sp.* and *Hyella sp.* are also found in lichenic form by reproduction in areas that are sufficiently moist and not exposed to sunlight [23]. In the study, a common algae flora was detected on the examined stone surfaces. In addition to moving algae samples such

as *Oscillatoria* (band 20), immobilized forms such as *Anabaena* (band 23) were also observed. Arid rock surfaces are generally Cyanophyceae species, whereas in humid areas *Gloeocapsa* sp. (band 6) and *Nostoc* sp. (band 21) covers the members. In blue-green algae, *Anabaena* sp., *Nostoc* sp., *Cylindrospermum* sp. genus such as (examples of the entire Nostacaceae family) provides fixation of atmospheric nitrogen [4, 23]. Thus, stone surfaces carrying conditions which are not suitable for microorganism growth become relatively suitable environments for microbial growth.

*Arthrospira platensis* PCC 7345 strain in band 8 is a thread-like, spiral-shaped prokaryotic organism using high-protein nitrogen-free non-nitrogen fixing [31]. Many such cyanobacterial species possess nitrogenase enzymes and can fix atmospheric nitrogen for their development [29].

Among the Cnobia classes, the majority of the inhabitants adhering to the rock and stone wall surfaces are generally dominated by biomass in the round, colored, sticky layer forming types. These cyanobacteria, which are capable of producing organic acids, further increase the rate of stone disintegration by providing organic nutrients for other bacteria and fungi [27]. *Gloeocapsa* sp. PCC 7428 strain and *Microcoleus* sp. are blue-green algae found in all natural environments (air–water–soil) because it has extremely colored dense gelatinous sheaths, it is easy to adhere to the stone surfaces and this coating causes colony formation on the stone surfaces even in dry weather conditions [27].

*Gloeocapsa* sp. and *Scytonema* sp. genus of blue-green algae lives in the presence of temperature, humidity and sun rays. They create a black shell on the stone surface and this causes the formation of black shell [32]. The predominant microorganisms of the black crust, indicative of microbiological degradation of stones; algae are the predominant microorganisms of green subaral spots, although they are fungi [33]. There are abundant *Chroococcales* (*Gloeotheca* sp., *Gloeocapsa* sp., Etc.) in biofilms formed in black shells [32]. In a study, it is stated that the mineral content of black shells is sufficient to support the development of cyanobacteria [27].

The habitat of these two species is followed by *Scytonema* sp., *Asterocapsa* sp., *Calothrix* sp., *Gloeocapsa* sp., *Chroococcus* sp., *Chroococcidiopsis* sp., *Cylindrospermum* sp., *Leptolyngbya* sp., *Blennothrix* sp., *Plectonema* sp., *Tolypothrix* sp., *Hassallia* sp., *Coleodesmium* sp. genus of microorganisms [21, 30]. *Cylindrospermum licheniforme* CCLA 995 strain detected in band 3 belongs to the group of Cyanophyceae. This species is an important member of the microbial ecosystem on the stone surface. In a study, a positive correlation was found between the elemental composition of the stones in the historical monuments and the communities of micro–macro organisms living on them. In this sense, a positive correlation was found between  $Mg^{+2}$ ,

$Na^{+}$ ,  $K^{+}$  elements, organic carbon, carbonate and chlorine ions and blue-green algae [34].

All species of Cyanobacteria formed on stone artifacts have nitrogen binding capacity and can survive under all conditions of the tropical environment, including deserts and salty environments [20]. Cyanobacteria and green algae living in the rocks increase the water retention capacity of the soil by disintegrating the stones into small pieces and forming soil [27]. The growth of cyanobacteria and algae results in the formation of shells on the stone surface, as well as in the microbial ecosystem pores on the rocks, resulting in increased stone colonization potential by keeping the stone moist for longer.

The *Brasilonema terrestre* strain CENA116 is a blue-green algae species commonly encountered in environments with high salinity on periodically wet rocks and walls [35]. In addition, *Spirulina* sp. (*Arthrospira*), *Oscillatoria* sp. and *Anabaena* sp. are fast-growing blue-green algae. In the studies on microorganisms on stone surface in historical monuments, *Brasilonema* sp. species have not been encountered from time to time. Most of the literature studies have focused mainly on European cyanobacteria.

*Microcoleus* sp., similar to *Anabaena Cylindrica* strains, *Chlorogloeopsis fritschii* PCC 6912 strain identified in the 16th band, develops in the presence of suitable organic matter in the stone environment and is involved in nitrogen fixation [36]. *Chitinophaga jiangningensis* JN53 strain can cause decomposition in potassium-containing rocks and allow the release of Si, Al, K and Fe. It shows positive reactions for oxidase and catalase activities [37, 38]. *Fischerella thermalis* strain PCC 7521 contributes to biodegradation by forming biofilms near the light source.

## Conclusions

In this study, by using several microbiologic analytical methods, the bacteria, fungus and cyanobacteria community that cause microbiological degradation on the stone surfaces of Yildiz Technical University Yildiz Campus and its surrounding areas were evaluated and their relations with each other and their contribution to stone biodegradation were identified.

The microorganisms have been cultivated at +4, 20, 25 and 30 °C. The *Alternaria* sp., *Penicillium* sp., *Cladosporium cladosporoides* species were identified at 4 °C degrees of temperature. At a development temperature of 20 °C for fungi; *Cladosporium sphaerospermum*, *Trichoderma koningii*, *Penicillium expansum*, *Scopulariopsis brevicaulis*, *Alternaria alternata*, *Ulocladium alternaria*, *Cladosporium cladosporoides*, *Aspergillus flavus* var. *columnaris*, *Penicillium steckii*, *Mucor* sp., *Geotrichum* sp., *Alternaria* sp. *Mushrooms* were determined, and 30 °C; *Ulocladium*

*alternaria*, *Cladosporium cladosporoides*, *Cunninghamella ramose*, *Penicillium expansum*, *Geotrichum sp.*, at the 25 °C *Penicillium frequentani*, *Penicillium expansum*, *Geotrichum sp.* Mushrooms have been identified. Via conventional cultivation methods, 17 fungal species, 9 fungal genus, and 4 species and 2 genus bacteria were identified. It was determined that the microorganism lived between 300 and 700,000 CFU in one gram of stones, the most microorganism lived in Cukursaray and at least in Yildiz Palace Fence Pavilion. It is seen that the dependence of microorganisms living on stones especially comes from water to the fore. It was determined that the number of microorganisms was low in June-July period when rain was low and it developed much more on stone surface in March and December period.

The *Cladosporium sphaeraspermum*, *Trichoderma koningii*, *Penicillium expansum*, *Scopulariopsis brevicaulis*, *Alternaria alternata*, *Ulocladium alternaria*, *Cladosporium cladosporoides*, *Aspergillus flavus var. columnaris*, *Aspergillus niger*, *Penicillium steckii*, *Cunninghamella ramosa*, *Penicillium frequentani*, *Penicillium sp.* genus fungi have been developed on the stone surface. Almost all of these fungi cause biodegradation of the stones, but also by entering the surface of the stones in the historical artifacts and discoloration, *Bacillus subtilis* bacteria form rough biofilms and color changes on stone surfaces due to this biofilm are visible to naked eye.

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## Compliance with Ethical Standards

**Conflict of interest** All authors declare that they have no conflict of interest.

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