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PROFILING THE BACTERIAL DIVERSITY IN HISTORIC LIMESTONE FROM ANAZARBOS ARCHAEOLOGICAL SITE BY ADVANCED MOLECULAR AND SPECTROSCOPIC TECHNIQUES

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ABSTRACT

The architectural remains as well as sculptures, mosaics, and other artifacts in the archaeological sites are mostly made of stone and susceptible to biodeterioration by microorganisms. Bacterial communities are known to have the most effective role in biological deterioration in stones. The ancient city of Anazarbos (Anavarza), chosen as the study area, is the 1st Degree Archaeological Site in the vicinity of Dilekkaya Village, Kozan District, about 70 km north of Adana. The microbiological, chemical, mineralogical, and microstructural properties of stone samples taken from different places were studied in this research. Samples were taken from i) where no deterioration occurred, ii) where different forms of deterioration were observed, iii) archaeological deposits, iii) archaeological deposits that came from deteriorated stone samples area. Spectroscopic, thermal, and microscopic techniques applied included: X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy-Energy Dispersive System (SEM-EDS), Thermogravimetric Analysis/Differential Thermal Analysis (TG/DTA). The bacterial biodiversity was analyzed by the application of Illumina-based next-generation sequencing methods. Results show intense biological colonisations with clay minerals on limestone surfaces. A patina of clay minerals was observed on newly excavated stone surfaces, while biological colonisations have not yet intensified. The metabarcoding analysis showed 15 bacterial phyla. The *Proteobacteria* and *Actinobacteria* were the most abundant phyla in both stones and archaeological deposits samples. Human activity (intensive agriculture, animal husbandry), accumulation of rainwater in excavated areas adversely affects stones, which leads to acceleration of biological deterioration in stones. Thus, all features of the site require multi-faceted studies prior to unearthing of archaeological remains.

KEYWORDS: bacterial diversity, stone deterioration, biodeterioration, archaeological site, Anazarbos, Anavarza

1. INTRODUCTION

In archaeological sites, stone material, especially above the soil, degrades due to exposure to weather conditions, air pollution, improper use, lack of maintenance, past interventions, and restorations that damage the structure. In addition to these, global climate change, as an emerging threat of the century, has critical impacts on heritage materials. Different agents may result in different types of decay patterns including the loss of stone material, detachment, fissures, and deformations (Doehne and Price 2010; Lee and Yi 2007; Scherer 2006; Camuffo 1995; Moussa 2019). Those that originate from microorganisms in stone deterioration are named as biodegradation in the general frame. The organisms causing the deterioration in stone are mainly bacteria (especially belonging to *Actinomycetes* and *Cyanobacteria* groups), archaea, fungi, algae, and lichens. The interaction between these organisms can lead to the deceleration or acceleration of the stone deterioration. Microbial communities can heavily colonize on stone materials, which may result in discoloration, colored patinas, pitting, erosion, cracks, and even severe damages. These organisms easily multiply within the fractures and pores of the stone. As such, they accelerate physical deterioration by applying pressure as well as chemical deterioration by creating an acidic environment (Urzi and De Leo 2001; El-Derby 2016; Liritzis et. al 2021). In addition, bacteria and fungi can penetrate the materials and cause severe damage due to metabolic functions, enzymatic activity, and mechanical attack (Sterflinger et al. 2013). Bacterial communities have the most effective role in biological deterioration in stones. Biological processes are considered irreversible, biodeterioration leads to structural and aesthetic damage that modifies the sustainability of the building (Ciferri 2002; Balland-Bolou-Bi et al., 2016). On the other hand, biofilm formation on stone surfaces as the result of microbial activities might be deteriorative and protective as well, and thus their physical and chemical interaction with the stone substrate must be studied in detail (Dornieden et. Al., 2000).

Within this framework, the aim of this research is to trace the bacterial diversity in historic stone material, together with chemical agents causing deterioration, tested through limestone samples in Anazarbos (Anavarza) archaeological site in Turkey. Having a significant historic background, the site offers a proper case by having stone monuments that are both long-term exposed to climatic conditions and recently unearthed. Lack of research on material deterioration and conservation in the archaeological site and obtaining legal permissions are the other motivation factors for the research. The study focuses on the microbiological, chemical, mineralogical, microstructural and thermal properties of the stone samples taken from places both where no deterioration and different forms of deterioration are observed, and from archaeological deposits. The above-mentioned properties of stones are determined by culture-independent advanced molecular techniques, XRD, FT-IR, SEM-EDS, and TG/DTA analysis, which are then compared.

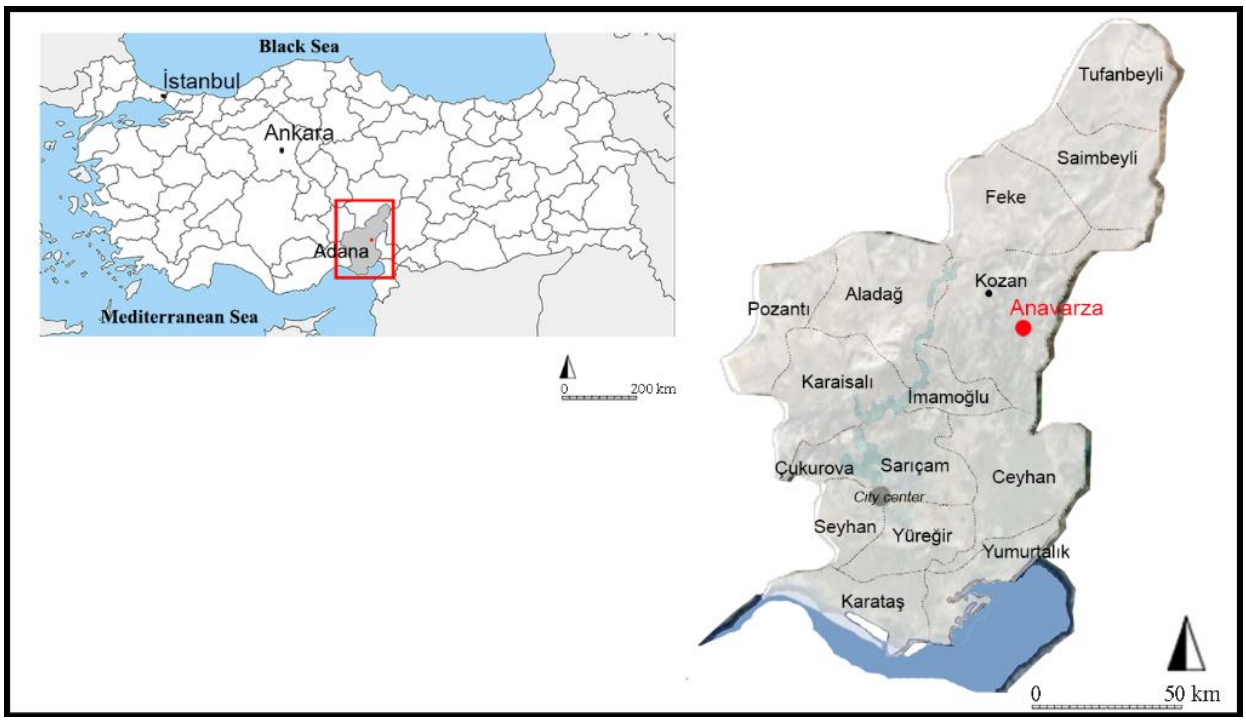

Figure 1. The location of the Anazarbos

2. MATERIALS AND METHOD

2.1. Site Description and Sampling

This study presents characteristics of limestones in the Anazarbos archaeological site and diagnosis of deterioration regarding the lack of studies identifying limestones and damage problems including biodeterioration. Inscribed in the UNESCO World Heritage Temporary List in 2014 (Yüceer et al., 2021), the ancient city of Anazarbos is situated in a rural area that is 70 km northeast of Adana in southern Turkey (Fig. 1). Although a part of the ancient city is inhabited by more recent village of Dilekkaya since the beginning of the twentieth century, several architectural structures of the city allow to trace its plan and understand the interventions made throughout ages including Roman, Byzantine, Armenian, Arab periods (Yüceer, et. al 2021). The main structures are the cordo, theatre, stadium, the monumental gate, aqueducts, rock tombs, the necropolis, mosaics, the Medieval Castle, bath and church ruins. These structures were built with limestones quarried from the nearby rocky masses (Ergeç 2001; İpekci et al., 2020; İpekci et al., 2021).

Among various significant architectural heritages, monumental Ala Gate (South Gate, Alakapı), dating to the end of second century, is the one that partially standing and thus visible from the main village road (Gough 1952) (Fig. 2a). It is the main gate to the city apart from the four gates in the Eastern Roman period walls surrounding the city (Posamentir, 2011). The plan and facades of the Ala Gate were designed in the same way as the triumphal arches in the ancient city of Rome in the Roman Empire (Yüceer et. al., 2021). As such, it can be considered as the most monumental, the oldest, and the most important of the Roman Period (Gough, 1952). There is a colonnaded main street (cardo) with stone column remains on both sides starting from the gate to the north direction (Fig. 2b). Since the recent excavations mainly focus on these two structures, limestones samples were taken from Ala Gate and the columns constituting the cardo (Fig. 3).

Figure 2. a. The Ala Gate b. Aerial view showing the colonnaded main street and Ala Gate

Representative samples are taken from the standing weathered surfaces, recently excavated surfaces, and sound inner cores of the limestones (the original inner core) (Fig. 3). During sampling, depending on the state of the stone, by scrubbing, by chipping from surfaces, and by using cotton swabs samples were collected. Besides, the sound parts of the samples were also taken out from the inner cores of the collected samples to identify stone types and compare the mineralogical, chemical, and microbiological compositions of differences from stone surfaces. Next to stone samples, deposit (archaeological deposit) samples were taken nearby the stone columns (at a depth of approximately 30 cm) to determine their mineralogical, chemical, microbiological, and soluble salt content. Archaeological deposits which accumulate through human activities include evidence of past activities at the site and represent past environments (European Standard EN 17652, 2021). Thus, these archaeological deposits play an important role in the preservation conditions at the site. Microbiological studies were performed under sterile conditions.

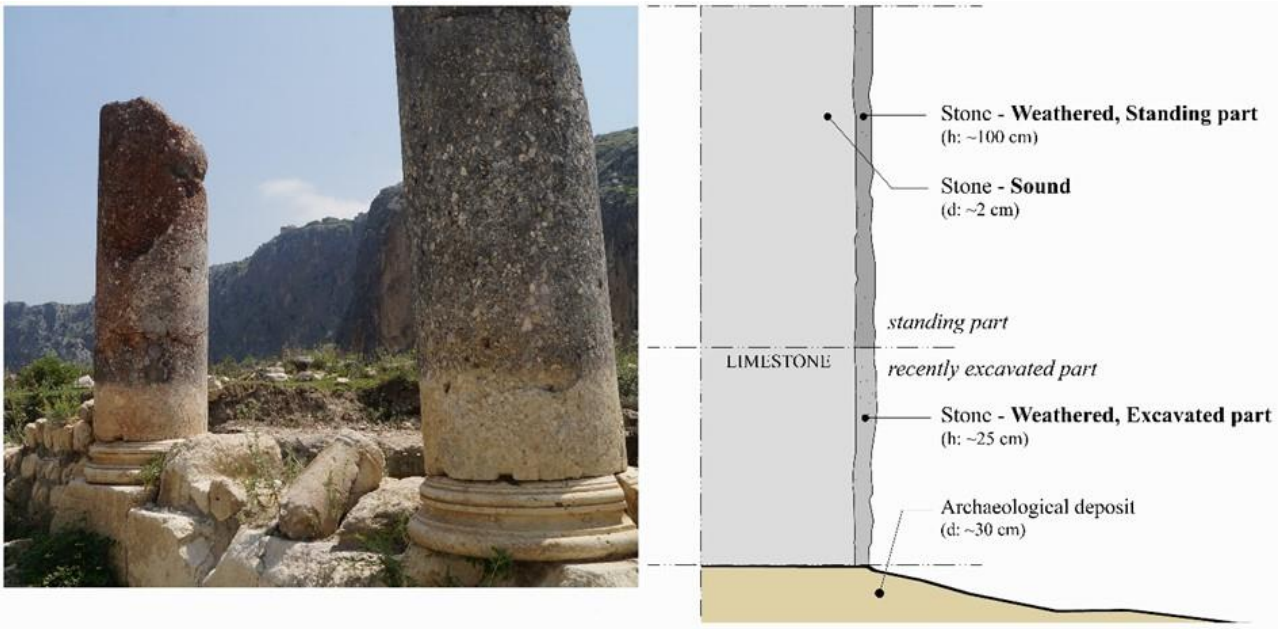

Figure 3. Figure showing where the samples collected

In total 11 limestone samples were collected: 3 samples from Ala Gate (Fig. 4) and 9 samples from the colonnaded road (Fig. 5). Nine archaeological deposit samples were collected: 2 samples nearby Ala Gate and 7 samples nearby the colonnaded road (Table 1). As described above, scraped and chipped samples were taken from the sound parts and surfaces of the stones. 11 samples were taken from the sound parts of the stones and 20 samples were taken from the stone surfaces. Among these surface samples, 17 samples were taken from the standing parts and 3 samples were taken from the excavated parts of the stones (Table 1).

Table 1. Limestone samples collected from the Ala Gate and stone columns and archaeological deposit samples

Sample	Definition
A.in.1, A.in.2, A.in.3,	Sound inner core samples from the Ala Gate (A)
A.st.1, A.st.2, A.st.3,	Scraped and chipped samples of the standing parts (height: 1.00 cm) from the Ala Gate (A)
C1.in.1, C2.in.1, C3.in.1, C4.in.1, C5.in.1, C6.in.1, C8.in.1, C9.in.1	Sound inner core samples from the 1st stone column (C1) to 9th stone column (C9)

C1.st.1, C2.st.1, C3.st.1, C3.st.2, C3.st.3, C4.st.1, C4.st.2, C4.st.3, C4.st.4, C5.st.1, C6.st.1, C7.st.1, C8.st.1, C9.st.1	Scraped and chipped samples of the standing parts (height: 1.00 cm) from the 1st stone column (C1) to 9th stone column (C9) surfaces
C5.ex.1, C7.ex.1, C8.ex.1	Scraped and chipped samples from the excavated parts (height: 50 cm) from the 5th, 7th, and 8th stone columns (C5, C7, C8)
A.de.1, A.de.2, C2.de.1, C4.de.1, C5.de.1, C6.de.1, C7.de.1, C8.de.1, C9.de.1	Archaeological deposit samples from where the stones were excavated

A: Ala Gate, C: Column, st: standing parts, ex: excavated parts, de: archaeological deposit

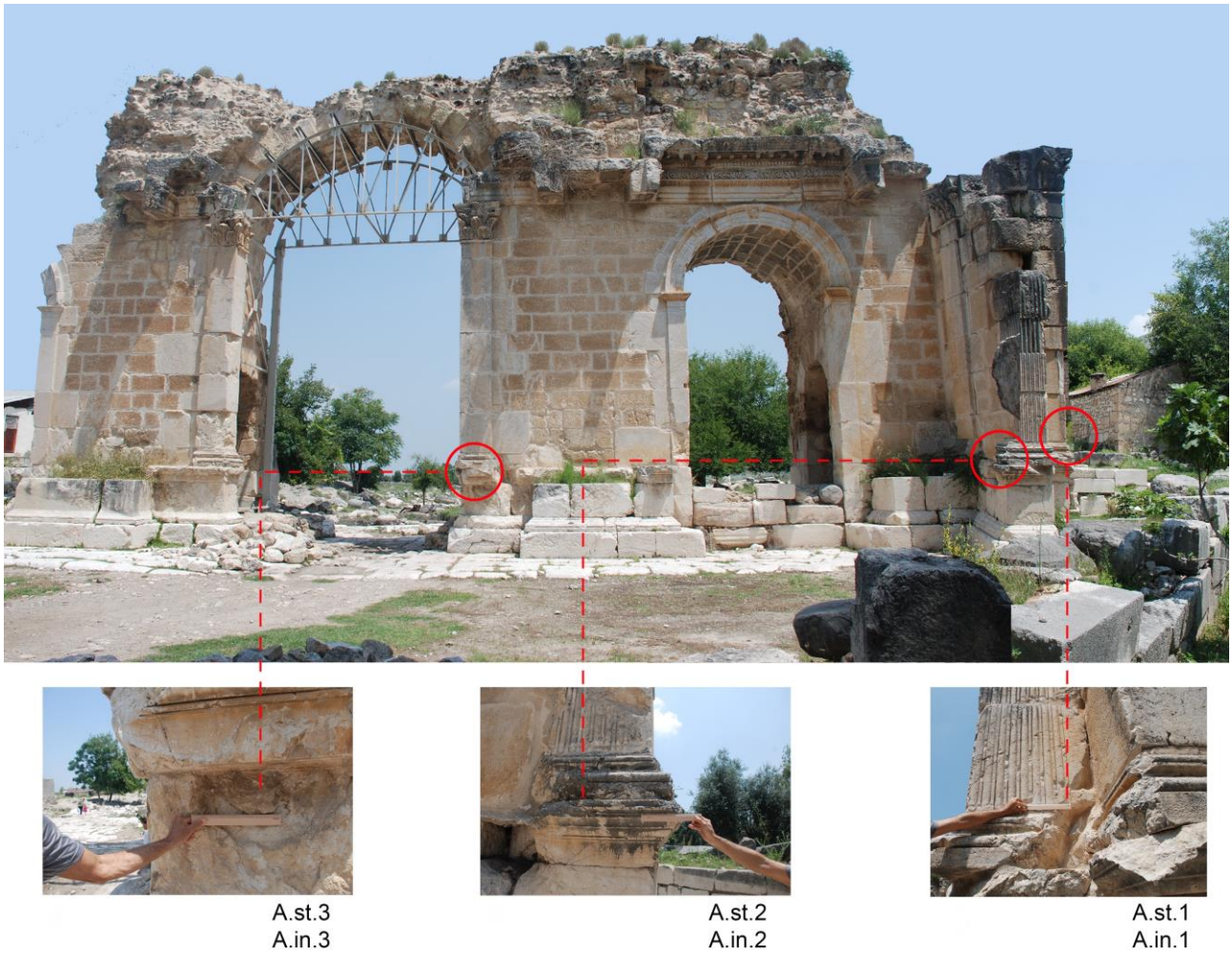

Figure 4. The positions of samples on Ala Gate

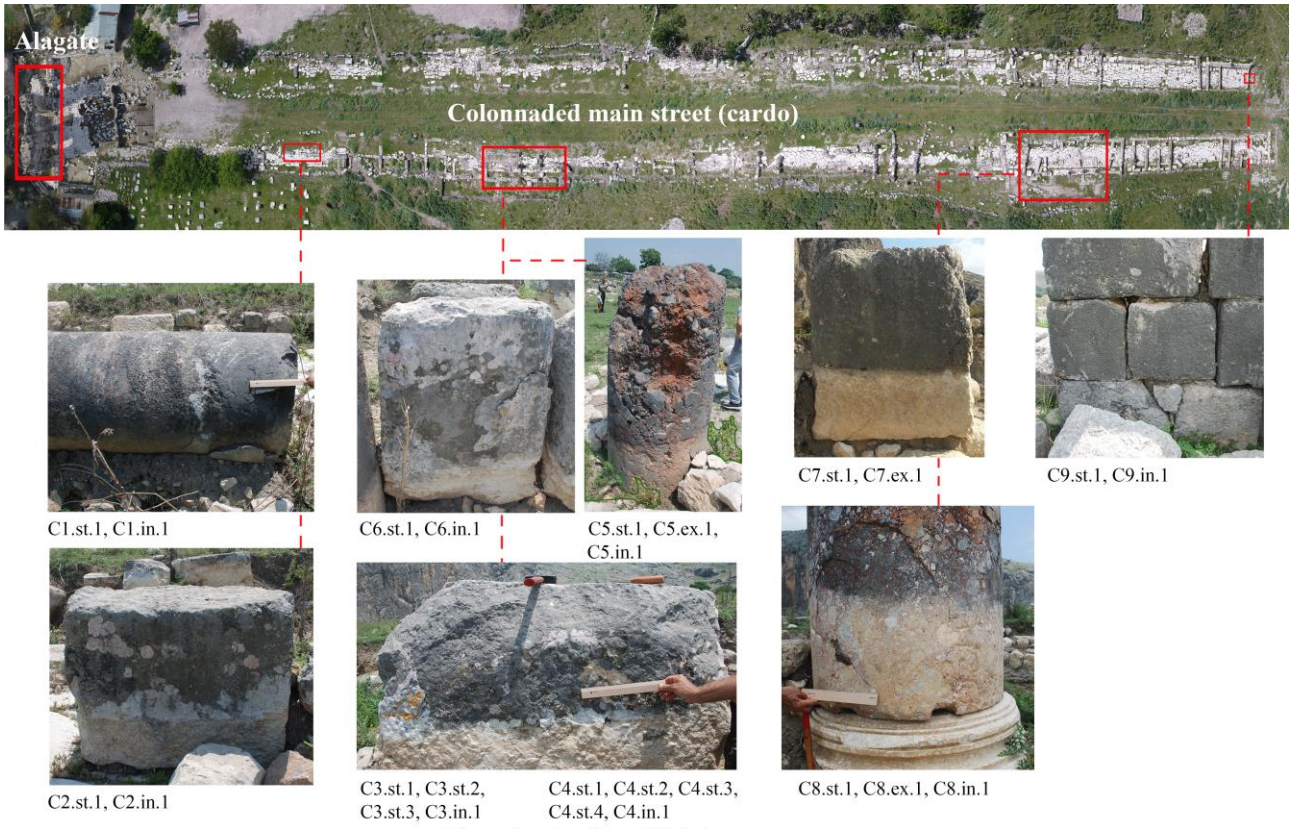

Figure 5. The positions of samples on collonnaded road

2.2. *Experimental methods*

The mineralogical compositions of representative samples of limestone were analysed by X-Ray Diffraction (XRD) and Fourier Transformed Infrared Spectroscopy (FT-IR). The chemical composition was identified by Scanning Electron Microscope-Energy Dispersive X-ray Spectrometry (SEM-EDS) and supported by Thermogravimetric Analysis/Differential Thermal Analysis (TG/DTA). Besides, XRD and SEM-EDS analysis were also performed on archaeological deposit samples to determine their mineralogical and chemical composition. Microstructural properties of the samples were identified by SEM-EDS and petrographic examination of thin sections of sound stone samples was determined by a petrographic microscope as well.

XRD analyses were applied on ground samples (particles less than 53µm grain diameter) by using a Rigaku Miniflex 600 with CuK_{α} radiation in the range of 5-70°, at a scan speed of 2 °/min. For the FT-IR analysis, the same ground samples were mixed with KBr (1 sample/10 KBr) and pressed into pellets under approximately 10 tons/cm² pressure. The analysis was performed on these pellets by using a Perkin Elmer-FT-IR System Spectrum BX spectrometer by scanning each of these samples four times. Thin sections were obtained from transversal and longitudinal cuts of small samples covered by epoxy resin, then, samples were observed in a petrographic microscope, and optical characteristics allowed the identification of minerals present in the stone. The chemical compositions of the samples were determined by Philips XL 30S-FEG Scanning Electron Microscope (SEM) equipped with Energy Dispersive System (EDS). Thermal analyses of the samples were carried out under a static nitrogen atmosphere at a temperature range of 25-1000°C with a controlled heating rate of 10°C/min, by using Hitachi brand STA7300 model TG/DTA. The soluble salt contents of the archaeological deposit samples were determined by an electrical conductivity meter (Black 1965). For percent soluble salt analysis, 1.00 g of finely-ground samples less than 53 µm were mixed with 50 ml distilled water. The conductivity of the filtered solutions was measured by the electrical conductivity meter (WTW MultiLine P3 pH/LF) and percent soluble salt contents were calculated.

2.2.1. Microbiological analysis of samples

Metagenomic DNA was extracted using the FastDNA®SPIN kit for soil according to the manufacturer's instructions (MP Biomedicals, Solon, OH). To amplify the variable V3-V4 regions of the 16S rRNA gene, the primers 341 F (5′-CCTACGGGNGGCWG-CAG-3′) and 805 R (5′- GACTACHVGGG-TATCTAATCC-3′) were used. MiSeq sequencing

adaptor sequences were added to the 5′ ends of forward and reverse primers. Approximately 12.5 ng of purified DNA from each sample was used as a template for PCR amplification in a 25 µl reaction mixture by using 2 × KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, MA, USA). For PCR amplification, the following conditions were followed: denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 sec., annealing at 55 °C for 30 sec. and extension at 72 °C for 30 sec., with a final extension at 72 °C for 5 min. Amplified PCR products were purified with Agencourt AMPure XP purification system (Beckman Coulter) and Nextera PCR was performed by using sample-specific barcodes. The constructed Nextera libraries were then sequenced by the Illumina MiSeq platform using MiSeq Reagent Kit v2 chemistry.

Sequence processing: Data was analyzed using the QIIME 2.0 pipeline (Caporaso et al., 2018). The pair-end 16S rRNA reads were first used cutadapt v1.9 program for the process of quality filtering, trimming and uploaded on the DADA2 (Callahan, B.J., et al, 2016) pipeline integrated into the Nephele platform (v.2.0, http://nephele.niaid.nih.gov)(Weber, N., et al., 2018).

3. RESULTS AND DISCUSSION

3.1. *Visual analysis of the weathering types of limestones*

A wide range of damages such as black crust, biological growth, discoloration, deposits, material loss, spalling, pitting, detachments, and cracks were observed on stones in the site survey. Among them, biological growth (higher plants/thriving micro-organisms) is a common decay type in the stones of the site (Fig. 6).

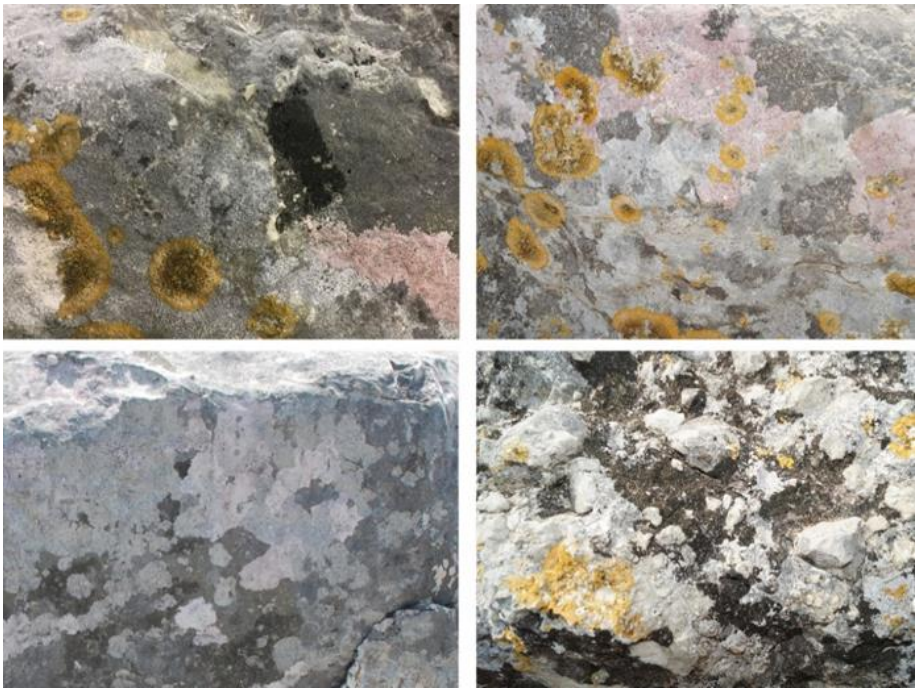

Figure 6. Thriving micro-organisms (lichens, algae, bacteria etc.) observed on stone surfaces

The severity of the damage is different between the standing part and the recently excavated part of the limestone columns. The standing parts of the stone surfaces are blackish, whereas the recently excavated parts are lighter in colour (Fig. 3). This colour change on the standing parts of the stone surfaces is related to microbiological colonization. The standing parts of the stone surfaces have been exposed to atmospheric conditions longer than the recently excavated parts, and thus, these parts have been heavily colonized by microorganisms.

Additionally, the surface temperatures of the stone columns are determined by a thermal camera. The surface temperatures of the standing parts are higher than the recently excavated parts due to the absorption of larger amounts of solar radiation which can be enhanced the stone deterioration by causing more stress within the stone by temperature changes (e.g. heating/cooling and wetting/drying cycles) (Garty 1990; Sand et al. 2002; Warscheid 2000).

3.2. *Characteristics of sound limestones*

Sound inner cores of limestones consist of micritic and sparitic fabrics with less fossil materials (Fig. 7). XRD patterns and FT-IR spectrums show that the sound limestones are mainly composed of calcite and quartz minerals (Fig. 8).

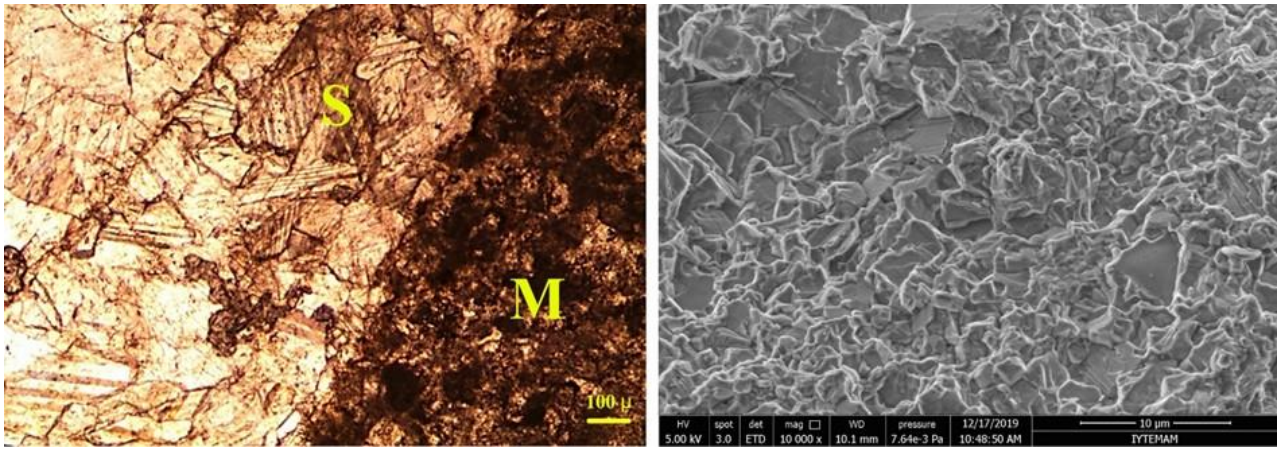

Figure 7. Thin section photomicrograph (S:Sparitic, M:Micritic) and SEM image of sound cores of lime-stone

According to SEM-EDS results, sound limestones are composed of a high amount of CaO (~92,5 %) and low amounts of SiO_2, Al_2O_3, MgO (Table 2). Thermal analyses (TG/DTA) of the sound limestones show that the weight loss between 600°C–800°C is nearly 43%. That weight losses correspond to the decomposition of calcium carbonate content, that nearly 95% in the limestone samples, in agreement with the SEM-EDS results.

Table 2. Mean values of major oxide compositions of limestones (13 samples) and archaeological deposit (9 samples)

	Na_2O	MgO	Al_2O_3	SiO_2	P_2O_5	SO_3	K_2O	FeO	CaO
Sound stone	0,10	1,67	1,06	3,72	0,15	0,26	0,21	0,29	92,50
	(0,06)	(2,62)	(0,54)	(2,66)	(0,11)	(0,26)	(0,09)	(0,12)	(4,10)
Standing surface	0,35	1,55	3,79	9,91	2,48	1,17	0,79	0,95	78,74
	(0,36)	(1,17)	(3,23)	(6,47)	(4,31)	(1,28)	(0,71)	(1,04)	(13,21)
Excavated surface	0,23	1,12	2,99	10,16	2,36	0,67	0,80	1,17	80,52
	(0,002)	(0,23)	(0,11)	(1,47)	(2,06)	(0,69)	(0,27)	(0,28)	(0,25)
Archaeological deposit	0,51	3,53	13,54	44,61	1,77	0,25	2,84	5,75	27,19
	(0,07)	(0,19)	(1,47)	(5,07)	(0,42)	(0,11)	(0,26)	(0,69)	(7,58)

3.3. Characteristics of limestone surfaces

Characteristics of limestone surfaces are examined by comparing standing and recently excavated surface samples.

3.3.1. Characteristics of standing parts of limestone surfaces

The results of mineralogical composition analysis carried out by XRD and FT-IR are shown in Fig. 8. In XRD patterns of standing parts of limestone surfaces, calcite, quartz, whewellite, and amorphous phase which are largely aluminosilicates peaks are identified. In FT-IR spectrums, calcite, silica, whewellite, O-H bands, and the organic compound bands showing the sign of biological tissues on the stone surfaces are observed (Fig. 8). The presence of whewellite indicates biological tissues as well because it is most probably formed by the reaction of calcite with oxalic acid produced by those biological tissues on the stone surfaces.

The results of chemical compositions (SEM-EDS) indicate that the amount of CaO decreases from sound inner cores to the surfaces of limestones whereas the amounts of SiO_2, Al_2O_3, and FeO increase (Table 2). The high amounts of SiO_2, Al_2O_3, and FeO on stone surfaces are due to the effect of degradation of the surface and more specifically to the accumulation of fine silt and clay minerals from the nearby surroundings. In addition, high amounts of SO_3 on some of the standing limestone surface samples could indicate the effects of air pollution due to SO_2 gases.

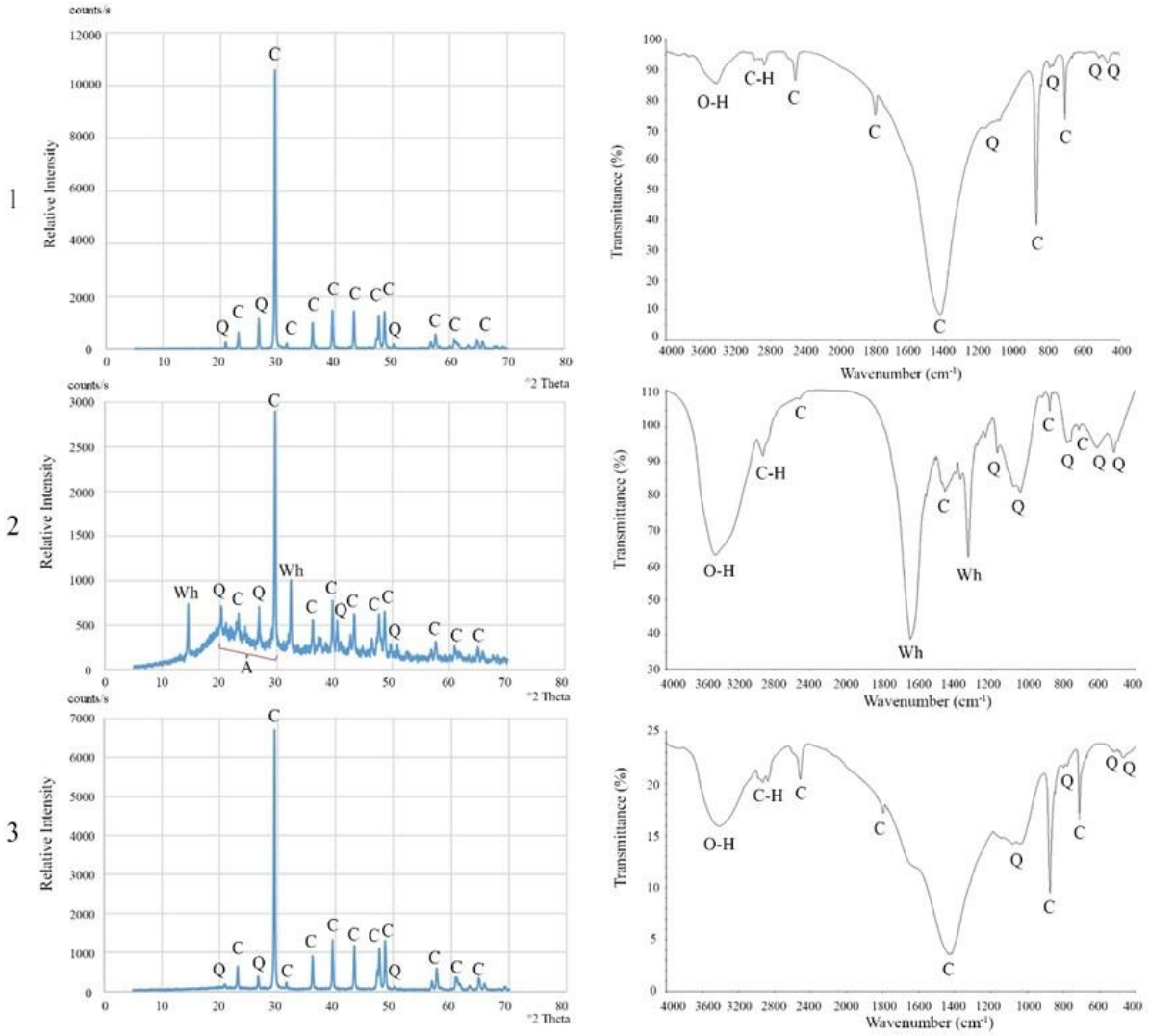

Figure 8. XRD patterns and FTIR spectras of the sound stones (1), the standing parts (2) and the recently excavated parts (3) of stone surfaces. [C: Calcite ($CaCO_3$), Wh: Whewellite ($CaC_2O_4.H2O$), Q; Quartz (SiO_2), A: Amorphous substances (Aluminosilicates), O-H: Oxygen-Hydrogen bands, C-H: Carbon-Hydrogen bands]

The thermal analysis supports the SEM-EDS analysis results. In the thermographs of the standing surface parts of the limestone, weight losses are observed in the range of 30–200 °C, 200–600 °C, and 600–900 °C, which are mainly due to absorbed water, decomposition of organic matter, and carbon dioxide, respectively. The high percentage loss at 200–600 °C and 600-900 °C for standing surfaces shows the presence of a high amount of organic matter due to biological colonization.

Microbiological colonization is well-developed throughout the inner cores of the stone. In the cross-section was evidenced biological colonization on the limestone varies between 100-200 µm. Their EDS mappings show higher amounts of C, Si, Al, and less amounts Ca than the sound inner core of the stone due to the stone colonizing agents (algae, lichens, fungi, bacteria, etc.) and some clay minerals (Fig. 9).

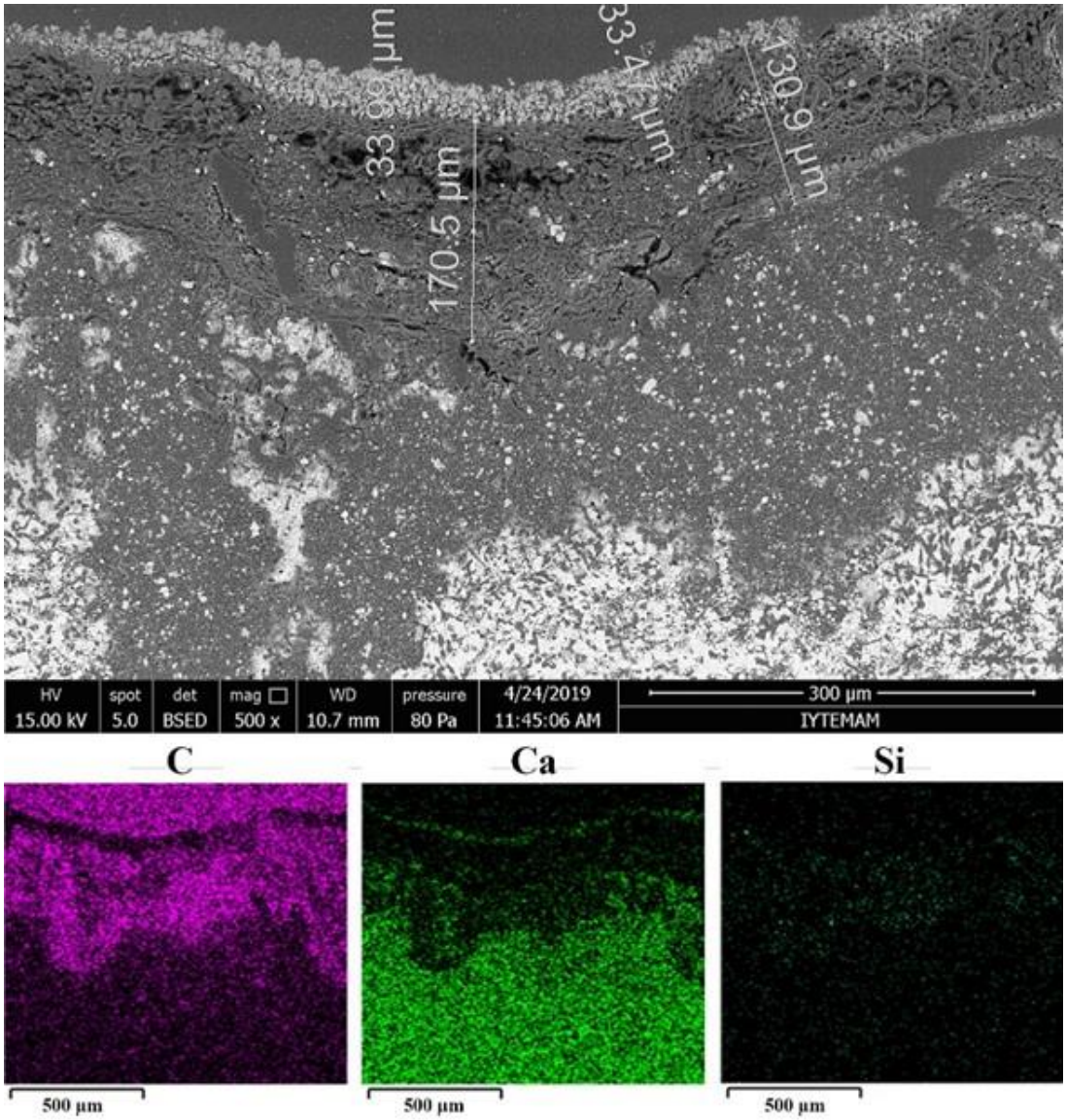

Figure 9. SEM image and EDS mapping of the intersection of the standing stone

3.3.2. Characteristics of recently excavated parts of limestone surfaces

XRD patterns and FT-IR spectrums of the recently excavated stone surfaces indicate that they are mainly composed of calcite and quartz minerals. Unlike the standing parts, recently excavated surfaces do not yet have whewellite minerals (Fig. 8).

The chemical compositions of the recently excavated stones contain higher amounts of SiO_2, Al_2O_3, P_2O_5, and FeO than inner sound parts of the stones, similar to the chemical composition results of the standing limestone surfaces mentioned before (Table 2). Besides, the high content of the P_2O_5 (especially sample C7.ex.1) could show the activities such as preparation, storage, and disposal of food and the fertilization of soil in the archaeological site (Herz and Garrison 1998). Fig. 10 shows clay accumulation varies between 50 and 200 µm on the recently excavated parts of the limestone surface (Fig. 10). That supports the presence of high amounts of SiO_2, Al_2O_3, and FeO on stone surfaces due to the accumulation of fine silt and clay minerals from the nearby surroundings. Clay minerals are not effective in buried stone deterioration, as the wetting and drying cycles under the soil are not as capable as on the surface. However, they cause rapid deterioration with the formation of wetting and drying cycles after the stone is unearthed (Thorn et. al., 2002).

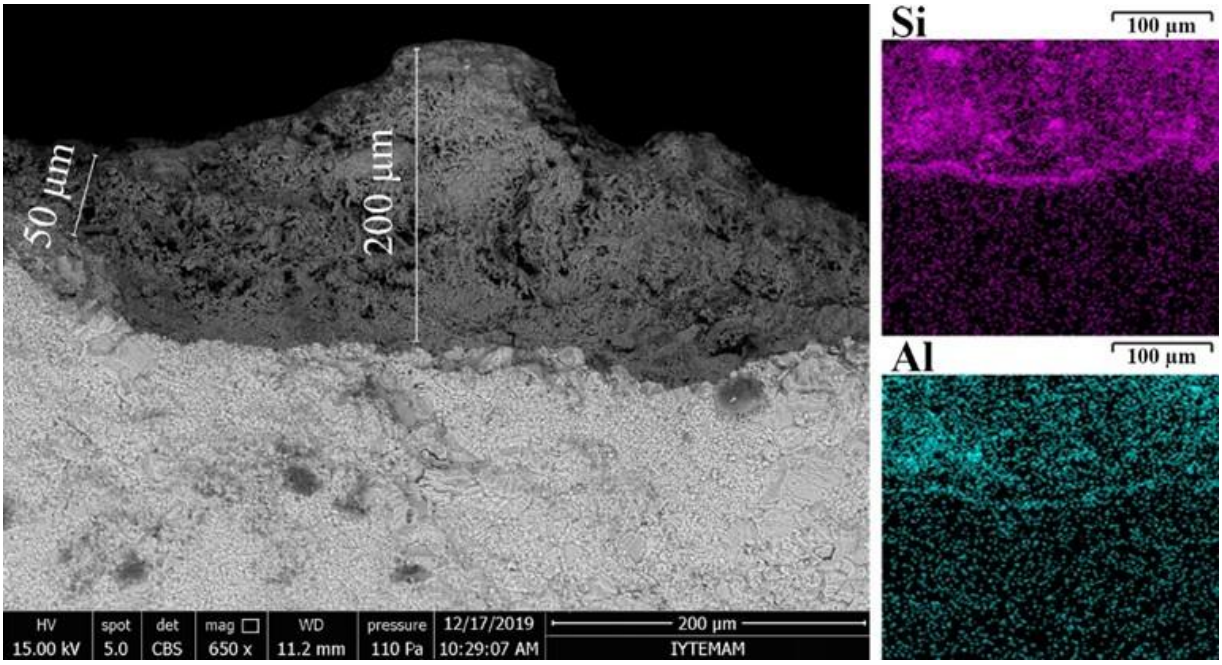

Figure 10. SEM image and EDS mapping of the intersection of the recently excavated stone surface

In the TG/DTA analysis of the recently excavated surface parts of the limestone, the lower percentage loss at 200–600 °C and 600-900 °C than standing surfaces are determined. It shows the presence the higher amount of organic matter due to biological colonization in standing parts of limestone surfaces.

3.4. Characteristics of archaeological deposit

Archaeological deposits are mainly composed of calcite and quartz, along with some feldspars (Fig. 11). SEM/EDS analyses indicated that they were composed primarily of SiO_2, CaO, Al_2O_3, and FeO. The minor elements were Na_2O, K_2O, MgO, and P_2O_5 (Table 2). Both of these results show that they are calcareous and siliceous archaeological deposit. The results of the electrical conductivity measurements indicated that archaeological deposits contain less than one percent of soluble salts. Therefore, it can be stated that soluble salts may be less effective in the deterioration of limestone columns.

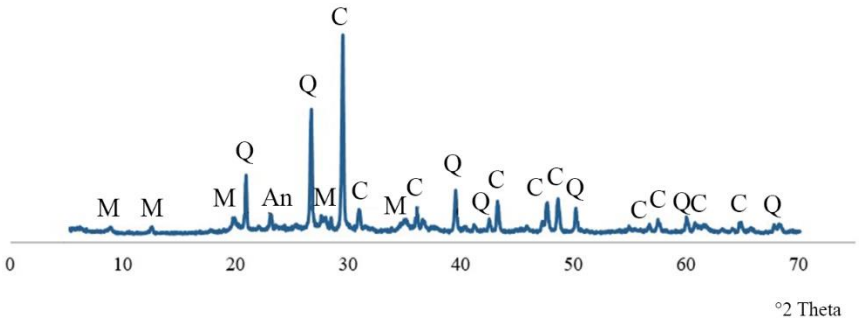

Figure 11. XRD pattern of archaeological deposit sample
[C: Calcite ($CaCO_3$), Q; Quartz (SiO_2), An: Anorthite, M: Muscovite]

3.5. Bacterial colonization of limestones

Over the past decades, with the help of the advent of cultivation-independent approaches has provided a wealth of information regarding bacterial community composition and their possible function in different habitats. In our study, a metagenomic approach was used to better understand the bacterial diversity present in each sample. Metagenomic methods have been gaining big popularity for the detection and analysis of microbial communities on ancient stone and monuments. The high-throughput sequencing results in our study revealed a very rich diversity of bacterial communities on the stone samples. We identified 15 annotated bacterial phyla and 195 bacterial genera in the samples (Fig. 12, Supp. Table 1). The *Proteobacteria* and *Actinobacteria* were the most abundant phyla in the analyzed samples (both stones and archaeological deposit), followed by *Firmicutes, Bacteroidetes, Acidobacteria, Cyanobacteria, Deinococcus-*

Thermus, Rhodothermaeota, Chloroflexi, Tenericutes, Verrucomicrobia, Fibrobacteres, Planctomycetes, Fusobacteria.

In the stones taken from the Ala Gate (A.st.1, A.st.3), *Actinobacteria* and *Proteobacteria* are dominant phyla and *Bacteroidetes* are slightly found as well (Fig. 12). In the stones taken from the columns (C1.st.1-C9.st.1), *Proteobacteria* and *Actinobacteria* are the most abundant phyla. Other abundant phyla belong to *Firmicutes, Bacteroidetes, Cyanobacteria,* and *Acidobacteria*. (Fig. 12).

Supplementary data for this article are available, please click to open Supp. Table 1.

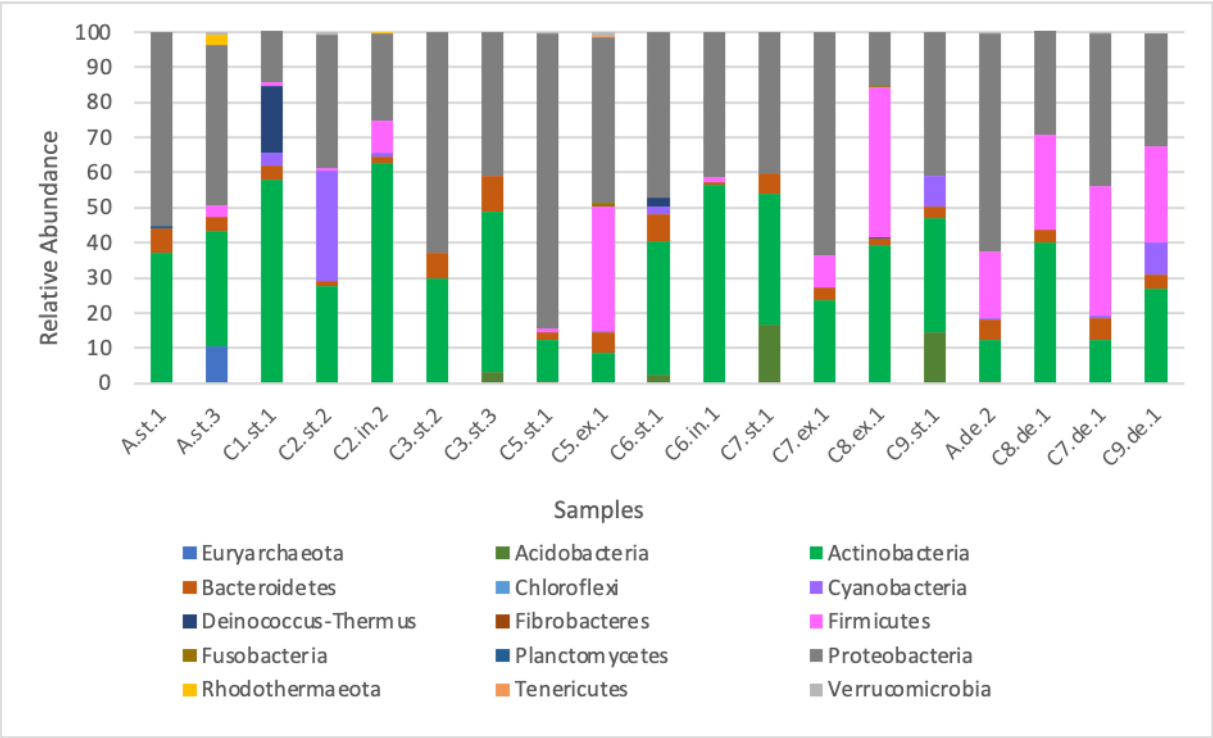

Figure 12. Relative abundance of distribution of bacterial phyla of the samples from Ala Gate (A.st.1-A.st.3), from the columns (C1.st.1-C9.st.1) and archaeological deposits (A.de.2-C9.de.1) (st: standing part, ex: excavated part, an inner part, de: archaeological deposit)

The 1st and 9th columns (C1 and C9) are covered by a black, greyish microbial formation. As shown in Fig. 12, sample C1.st.1 is mainly colonized by *Actinobacteria* (57,76%), with a contribution of *Deinococcus-Thermus* (18,8) and *Proteobacteria* (14,42%) respectively. The sample C9.st.1 is mostly colonized by *Proteobacteria* (41,05%) and *Actinobacteria* (32,43%), together with *Acidobacteria* (14,53%) and *Cyanobacteria* (8,45%).

As shown in Fig. 12, *Actinobacteria* is dominant in C1.st.1, C2.in.2, C6.in.1. There is a slight difference in bacterial distribution between samples from the deteriorated surfaces and sound inner cores of the stone samples (Fig. 12). The samples from the inner cores (C2.in.2 and C6.in.1) are mainly composed of *Actinobacteria* (higher than 50%) while the samples from the deteriorated surfaces (C2.st.2 and C6.st.1) are particularly different. Although the phylum of *Cyanobacteria* can resist high UV radiation making them among the most frequent microbial types on stone monuments, the phylum of *Cyanobacteria* was only dominated in the sample C2.st.2 (%31,62,) respectively. Researchers showed that *Cyanobacteria* have been identified in many stone monuments at sites with high humidity and illumination and also high porosity of stone matrix fissures (Sladana et al., 2018).

The 3rd column (C3) is covered by a very developed microbiological colonization throughout the inner cores of the stone. The sample C3.st.2 is taken from a yellow spot from this pigmented biofilm where lichens are observed and the sample C3.st.3 is taken from a pink spot where biopitting is visible on the surface (Fig. 13). As major bacterial population, the sample C3.st.2 is mainly colonized by *Proteobacteria* (62,98%), *Actinobacteria* (29,96%), *Bacteroidetes* (7,6%) while the sample C3.st.3 is colonized by *Actinobacteria* (46,02%), *Proteobacteria* (40,63%), *Bacteroidetes* (10,2%) (Fig. 12).

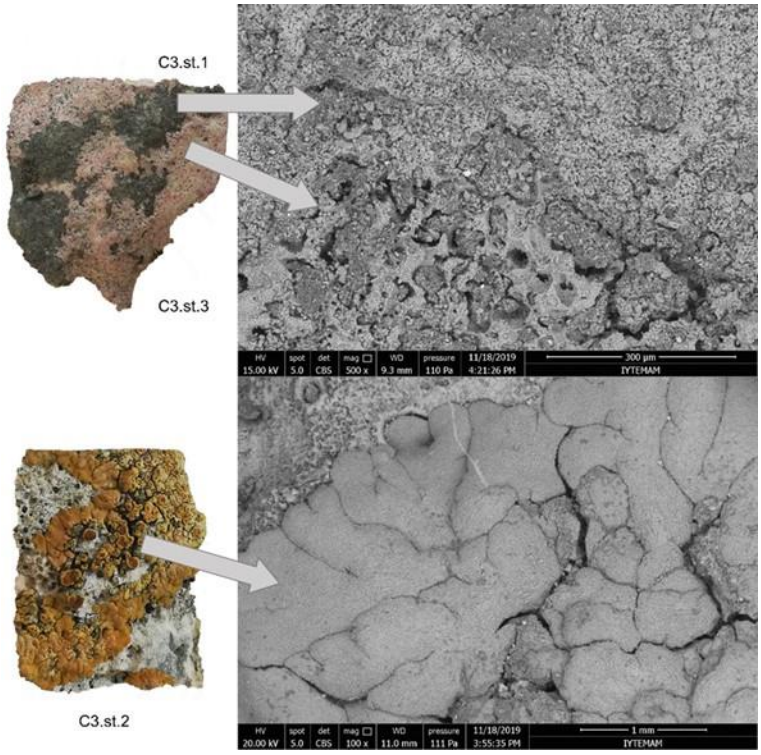

Figure 13. SEM images of the sample C3.st.1, C3.st.2 and C3.st.3

It was a clear difference between the excavated and unexcavated parts of the stone. Our study showed that bacterial growth developed at different severity on the standing part and recently excavated part of the columns. The standing parts of the stone surfaces have been exposed to atmospheric conditions longer than the recently excavated parts, and thus, these parts have been heavily colonized by microorganisms. The results show that bacterial populations between samples from the standing and excavated surfaces of the columns are different. The phylum of *Proteobacteria* is mainly found in the standing surface samples C5.st.1 (84,04%) and C7.st.1 (40,37%), as secondary group *Actinobacteria* is detected in C5.st.1 (11,64%) and C7.st.1 (37,33%). Unlikely, the *Firmicutes* are dominantly found in the recently excavated surface samples C5.ex.1 (35,57%), C8.ex.1 (42,75%) and slightly found in C7.ex.1 (9,25%), together with *Proteobacteria* and *Actinobacteria* members (Fig. 12). *Proteobacteria, Actinobacteria Firmicutes, Bacteroidetes,* and *Acidobacteria* are also frequently present on ancient Stones and ancient mural paintings. The genus *Bacillus* belonging to the phylum *Firmicutes* (Fig. 12) is identified frequently on these excavated surface samples in the area. The members of the genus *Bacillus* are a common inhabitant of soil, besides they can survive in other environments (such as water) as well as they are frequently found on stone monuments because of their resistance to extreme environmental conditions such as high temperature (Schereer et al. 2009, Daffonchio et al. 2000). The presence of organisms of the genus *Bacillus* on recently excavated stones may be generated from the accumulation of fine silt and clay minerals on their surfaces.

At the genus level, 3 bacterial genera are most abundant in these stones: the genus *Rubrobacter, Sphingomonas*, and the genus *Bartonella* (Supp. Table 1). The genus *Rubrobacter* is found in almost all stone samples (Supp. Table 1). The presence of the genus *Rubrobacter* could be associated with discoloration phenomena, especially related to the reddish discoloration of monuments (Ortega-Morales et al. 2004, Imperi et al. 2007). Additionally, *Rubrobacter* strains have a major role in both efflorescence formation and mineral precipitation and affect biological degradation by leading to detachment of mineral grains on biofilms (Laiz et al., 2009). The genus *Sphingomonas* is found in almost all stone samples, similar to the genus *Rubrobacter* (Supp. Table 1). *Sphingomonas* species can be detected in a wide variety of environments due to their ability to grow in low nutrient and water conditions (Pinhassi and Berman 2003). Besides, *Sphingomonas* are capable to produce yellow pigments on stone surfaces, which enables them to survive under high UV radiation (Mihajlovski et. al., 2017).

The genus *Bartonella*, another member of *Alphaproteobacteria*, is dominated most of the stones in the area (Fig. 10). *Actinobacteria* (e.g. *Rubrobacter*) and *Alphaproteobacteria* (e.g. *Sphingomonas*) have been detected in connection with stone damage phenomena in different studies. *Actinomycetes* play a key role in endoliths in ancient stone monuments, and many reports have

shown their ability to biodeterioration and degrade ancient archaeological monuments

Notably, a high proportion of *Bartonella japonica* species belonging to the genus *Bartonella* were detected in almost all samples (Sup. Table 1). These species use some mammals as reservoir hosts (Dehio et al. 2001). They may be transferred from animals to the stone due to animal husbandry activities nearby the stones. It has been shown that *Bartonella japonica* may have very important roles in the biological degradation of some historical stones by degrading airborne insecticides (Brenner et al., 1993; Birtles et al., 1995). Therefore, the presence of a high amount of Bartonella japonica may be related to the insecticide used due to intensive agricultural activities in the region.

Besides *Proteobacteria*, we also detected *Acidobacteria, Actinobacteria, Bacteriodetes, Firmicutes,* and *Cyanobacteria* in all archaeological deposits. The investigation between the archaeological deposits and the related stone samples which were recently excavated, it is showing almost the same bacterial diversity as the archaeological deposits. On the other hand, the bacterial diversity of the archaeological deposit samples and the biodeterioration parts of the standing stones were completely different from each other. These results reveal a very clear picture in the field of conservation. It shows once again that the stones should not be unearthed without taking full protection measurements in the area.

4. CONCLUSIONS

Stone deterioration is one of the research areas in the analysis and conservation of historic buildings and monuments that still open to further studies. Existing studies on this subject frequently focus on chemical deterioration while biological aspects and cross relations receive relatively less attention. Detection of deterioration in stones in Anazarbos is a pioneering study in Turkey with a focus on bacterial diversity that is among the main causes of biological degradation in archaeological areas. It also helps to reveal the bacterial communities that cause deterioration in limestone. As such, the data obtained from the project has become a guiding resource for scientific and conservation studies in archaeological areas.

The case of Anazarbos showed that the accumulation of rainwater and human activities (intensive agriculture and animal husbandry) in the excavation area harmed the stone artefacts by accelerating the biological deterioration. Since the main sources of income in Dilekkaya village and Kozan district, where Anazarbos ancient city is located, are agriculture and animal husbandry, the use of pesticides is quite common as is expected due to the high intensity of agricultural activity. During the study, some bacterial groups that decompose insecticides were found in some stones. This indicates that the stone material cannot be evaluated on its own, but abiotic, biotic, and human activities such as agricultural and livestock activities and stubble burning operations hugely a huge significant impact on stone biodeterioration. To this end, the study underpins the need of implementing preservation measures in cultural heritage sites via multidisciplinary work that takes into consideration the region's socio-cultural, economic, geographical, and other factors.

To prevent the biodeterioration of stone monuments should therefore be interdisciplinary, drawing on archaeology, physics, chemistry, microbiology, geology, and ecology. In addition, environmental factors and anthropogenic effects should be monitored and maintained within a range suitable for the ancient stone monuments while providing protection against colonization by microorganisms.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

Conceptualization, RA, HY.; methodology, RA, HY, HB.; software, -; validation, RA, MTD and Eİ; formal analysis, RA, Eİ, and MTD; investigation, RA, Eİ, HY, MTD, and HB.; resources, RA, Eİ, HY, MTD, and HB; data curation, RA, MTD, and Eİ; writing—original draft preparation, RA, Eİ,; writing—review and editing, RA, Eİ, HY, MTD, and HB.; visualization, RA, HY, Eİ, and MTD; supervision, HB; project administration, RA; funding acquisition, RA. All authors have read and agreed to the published version of the manuscript.

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