

Diversity and Community Structure of Biofilms Found on Stone Monument and Assessment of a Conservation Measure

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Abstract

The Leizhou area in the Guangdong province of China is famed for its unique folk art creation, the Leizhou stone dog monument, which is mainly made of basalt. Some of them have a history of more than two thousand years beginning from the Han dynasty. Since the stone dog is mostly seen in the outdoors, such as in fields, many of them have undergone biodeterioration due to lichen and fungi. This study explores measures to prevent biodeterioration and protect stone dogs from degradation. A portable microscope and SEM-EDS were used to observe and identify the micromorphology of the microbiology on the stone, and highthroughput Sequencing technique (Illumina sequencing of gene amplicons) was conducted to analyse the fungi diversity of the biofilm on the stone dogs. The results showed that the dominate fungi were *Caloplaca*, *Trebouxia*, *Chaetomium*, *Clitopilus*, *Acanthostigma*, *Tolypocladium*, *Aspergillus*, and *Toxicocladosporium* at the genus level. To control biodeterioration, three kinds of antimicrobials, BYS4002 mix BYB10073, KY-104, and Sino-307, were used for sterilization. After treating the samples for 2 years, BYS4002 mix BYB10073 was found to be the most effective resulting in no trace of lichen on the surface. Sino-307 was also good but revealed traces of lichen on the surface. KY-104 was found to have a very short bacteriostatic effect.

1. Introduction

Stone has for long been used as a medium for artistic expression, ranging from its use in the construction of ancient monuments and historic buildings to small-scale statues (Warscheid & Braams, 2000). The Leizhou stone dog is a unique folk art creation seen in the Leizhou area of Guangdong province in south China. Some have a history of more than two thousand years (from the Han dynasty). The stone dog, a traditional culture heritage of the Leizhou Peninsula, has played historical roles, varying from being used as a totem by tribes, as a mascot to guarding gods, to greeting statues, carrying with it the intangible value of folk carving art, a product of the combination and interaction of social historical factors and regional natural conditions. Roughly, about ten thousand stone dogs are located throughout the Leizhou Peninsula. The main material used to make them is basalt from the Leizhou Peninsula, which lies at E109°31'-110°55' and N20°-21°35' and has tropical monsoon climate. As most of the stone dogs are located in the outdoors, over a long period, in addition to sun exposure, rain erosion, other natural causes, such as environmental pollution, man-made damage, and other factors, biodeterioration caused by microorganisms is one of the important factors that has led to the degradation of the stone dogs. Microorganisms are known to promote the weathering of rocks through the biochemical disruptive pressure of the growing hyphae and the biochemical fractionation of mineral constituents (Money, 2001). While the weathering of rocks to soil formation is unquestionably essential for the evolution of life on earth, the decay of culturally significant stone artefacts represents an irretrievable loss of heritage and history. Hence, this article aims to examine the biodeterioration of stone dogs and propose ways to protect them.

2. Materials And Methods

2.1 Sampling Site and Samples

Stone dogs are seen in every village in Leizhou; they are subjected to high humidity and rainfall under the tropical climate. Hence, there is remarkable microbial colonization on stone dogs in different sites. Fig. 1 shows some of the sampling sites. And table 1 shows samples for fungal diversity analysis by Illumina sequencing of gene amplicons.

2.2 Observation by microscope

Different colour biofilms were observed using the DG-3X portable microscope (Japan) on the site. Some biofilm samples were collected and sent to the lab for observing them under a light microscope. A drop of each culture was transferred to a microscope slide and examined using Axio Imger A2 m microscope (ZEISS).

To perform scanning electron microscopy (SEM) (Quanata 650 FEI), small fractions of the stone slabs were separated and gold-coated prior to observation by SEM, and a semiquantitative elemental analysis of the mineral fraction of the speleothems was conducted by energy-dispersive X-ray spectrometry (EDS) (EDAX).

2.3 High throughput Sequencing technique (Illumina sequencing of gene amplicons)

2.3.1 DNA Extraction

DNA was extracted using DNA extraction kit for the corresponding sample. The concentration and purity were measured using the Nano DropOne (Thermo Fisher Scientific, MA, USA) .

2.3.2 Amplicon Generation

ITS genes of distinct regions (ITS2) were amplified used specific primer (GCATCGATGAAGAACGCAGC & TCCTCCGCTTATTGATATGC) with 12bp barcode. Primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA). PCR reactions, containing 25 µl 2x Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 µl each primer (10 mM) and 3 µl DNA (20 ng/µl) template in a volume of 50 µl, were amplified by thermocycling: 5 min at 94°C for initialization; 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 30 s extension at 72°C; followed by 10 min final elongation at 72°C. The PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA, USA).

2.3.3 PCR Products Detection, Pooling and Purification

The length and concentration of the PCR product were detected by 1% agarose gel electrophoresis. Samples with bright main strip between 250bp could be used for further experiments. PCR products was mixed in equidensity ratios according to the GeneTools Analysis Software (Version 4.03.05.0, SynGene). Then, mixture PCR products was purified with E.Z.N.A. Gel Extraction Kit (Omega, USA). Each project selects the appropriate primers for amplification. When the final primer sequence was not known, it could be viewed in the mapping file of the analysis result package.

2.3.4 Library preparation and sequencing

Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). At last, the library was sequenced on an Illumina Nova6000 platform and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology Co.,Ltd. Guangzhou, China).

2.3.5 Data analysis

Sequencing data processing

Fastp (version 0.14.1, <https://github.com/OpenGene/fastp>) was used to control the quality of the Raw Data by sliding window(-W 4 -M 20). The primers were removed by using cutadapt software(<https://github.com/marcelm/cutadapt/>) according to the primer information at the beginning and end of the sequence to obtain the paired-end Clean Reads. Paired-end clean reads were merged using usearch-fastq_mergepairs (V10,<http://www.drive5.com/usearch/>) according to the relationship of the overlap between the paired-end reads, when at least 16 bp overlap the read generated from the opposite end of the same DNA fragment, the maximum mismatch allowed in overlap region was 5 bp, and the spliced sequences were called Raw Tags. Fastp (version 0.14.1, <https://github.com/OpenGene/fastp>) was used to control the quality of the Raw Data by sliding window(-W 4 -M 20) to obtain the paired-end Clean Tags.

OTU cluster and Species annotation

For each representative sequence, Unite (for ITS, <http://unite.ut.ee/index.php>), database were used to annotate taxonomic information by usearch -sintax(set the confidence threshold to default to ≥ 0.8). The taxonomy of the species annotation was divided into seven levels: kingdom (L1), phylum (L2), class (L3), order (L4), family (L5), genus (L6) and species (L7).

A rarefaction analysis and coverage were applied to estimate the representation of the phylotypes and to characterize the microbial diversity of these samples. The rarefaction curves were produced with the usearch-apha_div_rare (V10 <http://www.drive5.com/usearch/>) .

Phylogenetic relationship Construction of all Samples

In order to study the difference of the dominant species in different samples(groups), the OTU representative sequences with the relative abundance in the first 20 were conducted using the FastTree software, and the relative abundance of each OTU and the species annotation information of the representative sequence were combined with the ggtree software package for visual display. Using the anosim, MRPP, Adonis and AMOVA of vegan and pegas package in R software to analyze the difference of community structure between groups and whether the differences were significant.

The differences between groups were analyzed by alpha diversity index using R software, would be carried out with parameter test and non parameter test respectively.

2.4 Antimicrobial treatments

Three kinds of antimicrobials (Table 3) were used in the sterilization experiments on the surface of the stone dog (S678). An area covered with biofilm on the surface of the stone dog was divided into 4 small areas (Fig. 2), brushed with different reagents, and the effects were assessed after 1 day and after 2 years.

3. Results

3.1 Chemical composition of stone dog

The chemical composition of the stone dog is as shown in Table 3. The stone dog is made of basalt, which is mainly composed of silica. Basalt density is 2.8-3.3 g/cm³; it has great compressive strength, nearly 300 MPa or even higher. Basalt also has high durability, but it is brittle. XRD analysis shows that the mineral composition of basalt is basic plagioclase including Labradorite ($\text{Ca}_{0.65}\text{Na}_{0.35}\text{Al}_{1.65}\text{Si}_{2.35}\text{O}_8$), anorthite ((Ca,Na) (SiAl)₄O₈), a small amount of albite (NaAlSi₃O₈), Augite, $\text{CaMg}_{0.74}\text{Fe}_{0.25}\text{Si}_2\text{O}_6$, and some amorphous substance.

3.2 Microscopy observation

For this study, samples were taken from different stone dogs. All sampled sites exhibited obvious colonization by microorganisms, which showed different colours, such as green, black, brown, yellow, and white. The microorganisms detected microscopically in the biofilm communities are as shown in Fig. 3. The organisms attached to the surface mainly include lichens, fungi, algae, and mosses. On observation through the light microscope, although small, single-celled bacteria were seen in large numbers in all cases, filamentous cyanobacteria constituted the main biomass, as shown by direct examination of the biofilms (Fig. 3). SEM revealed an abundance of hyphae masses and the state of hyphae attachment to the surface of the stone. EDS results show that the hyphae attachment to the rock had a high content of Aluminium (Al) (Fig. 4).

3.3 Analysis of fungal community structures

The coverage of eukaryotic libraries was all above 96%, indicating that the major of the biofilm diversity in the clone libraries was detected. In addition, the rarefaction curves generated from our clones reached the asymptote (Fig. 5), indicating that the diversity in the libraries was representative of the community and there was no need for further sampling of more clones.

Most of the seven samples were grouped in fungi, only a few belonged to *Chlorophyta*. Almost all fungal communities were composed of *Ascomycota* and *Basidiomycota*. These samples were taken from different stone dogs. M4 had the highest content of *Ascomycota* (83.7%) and *Chlorophyta* (5.74%).

Taxonomic composition of the different samples communities appeared to be different. In this study, the dominate fungal communities on the genus level in M1 were *Chaetomium* and *Clitopilus*, in M2 was *Acanthostigma*, in M3 was *Tolypocladium*, in M4 were *Caloplaca* and *Trebouxia*, in M5 were *Caloplaca* and *Acanthostigma*, in M6 were *Aspergillus* and *Toxicocladosporium*, in M7 were *Coniosporium*. And cluster tree was established by clustering analysis of samples under classification on genus level in Fig. 6.

Clustering and heat map analysis visualizing the OTUs present in each sample were conducted to examine the effect of the sample origin (collection place) on the fungal communities (Fig. 7). The fungal communities of 6 samples, namely, M1, M2, M3, M5, M6, M7, were similar, and M4 were not similar. Thus, the heat map showed that there was special relationship existed between the fungal community structures and sampling origin.

3.4 Antimicrobial treatments

The stone dog's outer surface was divided into four areas, and three kinds of antimicrobials were brushed on the different areas. After 1 day, the green biofilms of the antimicrobial treated areas showed bleaching and dyeing (Fig. 8). This suggested that all three antimicrobials were effective against the biofilm, and there were no significant differences among them. However, after 2 years, the areas 5-2 and 5-4 showed obvious green biofilms, while areas 5-1 and 5-3 did not. Hence, it could be inferred that material 1 and material 3 could inhibit lichen growth. The BYS4002 mix BYB10073 was in fact found to be the most effective antimicrobial agent.

4. Discussion

Microbial biofilms are a common feature seen on stone in many parts of the world; these are dominated by microbial fungi, algae, and lichens (symbioses of fungi and algae) (Gorbushina, 2007). Under investigation, different colour biofilms were observed on the surface of stone dogs through micromorphology, which caused the different colour bio-pittings. Most of the bio-pittings were black and caused mainly by black fungi (Sterflinger & Piñar, 2013), which form small black colonies on and inside the stone and often occur in close association with lichens (Sterflinger, 2006). Some biofilms were orange or green; in green biofilms, the photosynthetic pigment could be observed (Fig. 3-J), which is used for photosynthesis to provide nutrition for fungi. In this study, fungi and lichen diversity maybe more or less the same in the different stone dogs, because, in general, under the same climatic conditions, microbial communities do not vary significantly among the different types of sandstone sampled (Nick et al., 2013), as is the case with basalt. The analysis data by Illumina sequencing of gene amplicons could lead to only a preliminary conclusion: *Caloplaca*, *Trebouxia*, *Chaetomium*, *Clitopilus*, *Acanthostigma*, *Tolypocladium*, *Aspergillus*, *Toxicocladosporium* and so on, were found to be the dominant communities in the biofilms on the stone dog. These are common fungus and *Chlorophyta* that cause biodeterioration, especially *Ascomycota* is the main community often forming mycelia in the porous space of the stones, in the study, the content in M4 was 83.7%. Molecular techniques for studying

microbial community diversity are well documented; for example, Nick (2013) studied eukaryotic microbial communities on sandstone building, which revealed that over 95% of sequence reads in the samples were from *Ascomycota*, and that the abundant taxa from *Ascomycota* were either sooty moulds or lichenised fungi. *Caloplaca* comes in a variety of colours, mainly yellow to orange red or rust red, and a few species white to black.

It is known that fungus and lichen can influence mineral and rock breakdown processes through both physical and chemical action. One of the important physical processes is the penetration of hyphae through intergranular voids and mineral cleavage planes, which can affect the structure of the rock surface (Chen et al., 2000). On the one hand, the surface of the stone could disaggregate directly, while on the other, pores could appear on the stone surface and make the stone surface lose its hydrophobic properties due to the presence of hydrated fungal biofilms. which could induce and accelerate other forms of physical weathering. Fig. 3-H shows the state of hyphae adhering to the stone, indicating how the surface structure of the stone dog was changed by the fungi and the lichen.

However chemical action is a complex and long-term process. As pioneer colonizers, lichens can create a favourable microenvironment by increasing the bioavailability of mineral elements and nutrients to successive life-forms, and this process mainly involves the dissolution of important mineral elements (Si, Al, Mn, Fe, Ca, K) leading to the biodeterioration of the stone. And hence, Mn and Fe may be involved in the bio-chemical action of the microorganisms leading to biodeterioration of the stone. EDS microanalysis of green biofilm reveal high content of Al. Jones et al. (1980) report that in the colonized basalt, lichen growth resulted in the formation of ferromagnesian minerals and made the surface calcium-rich, further led to deposition of iron and magnesium and release of calcium and aluminium from the primary minerals, and to the formation of amorphous materials(Chen, 2000).

For conservation of the stone dogs, the first step is to remove or kill the microorganisms on the stone. Microbial colonization on stones depends on environmental factors, such as water, pH, temperature, light, nutrient sources, and other parameters, for example, mineral composition (Warscheid & Braams, 2000; Lan et al., 2010). Hence, controlling any one of these factors can prevent microbial colonization growth. However, most stone dogs are placed outdoors, and temperature and sunlight cannot be controlled; therefore, the small part of the stone surface environment that had to be studied had to be altered. Water is a major limiting factor for most microbial biofilms. When water content on a stone surface is kept low, it could affect biofilm formation (Gorbushina, 2007; Liu et al., 2018). Therefore, choosing a water-repellent material as an antimicrobial agent may be effective. Among the antimicrobial treatments, Material 1 seemed to work well maybe because it was added to an anti-weathering material to provide a germicidal effect. The water-repellent material forms a sealing film on the stone surface and prevents water from seeping into the stone, and the dry stone surface makes it difficult for the microorganisms to survive. In the study, biocide was also used; it can kill biofilms at the early stage itself, but its effect does not last. This could be because the microorganisms quickly develop resistance or become adapted to this biocide.

Evaluation of the effectiveness of the treatment, there were no other additives or elements other than basalt detected by portable XRF after treatment, and no other impact was found. But conservation of stone monuments is a long-term work, therefore, detailed evaluation is needed for antimicrobial treatments on stone relics, including the influence of these not only on the stone body, such as colour changes, density, and porosity variation by measuring water absorption and so on, but also on the environment, such as a chemical attack contaminating the atmosphere or the water. However some reports were not recommended to applications of biocides (Liu, et al., 2018; Gu & Mitchell, 2013). But when biodeterioration begins to destroy a structure, it is necessary to consider eliminating the microorganisms using biocide treatment, otherwise, microorganism activity can lead to serious biodeterioration of any stone structure (Warscheid & Braams, 2000). A more comprehensive approach is required to estimate the application of water repellents or biocides.

Conclusions

Stone dogs undergo degradation due to the biodeterioration caused by lichen and fungi. On investigating the microbial communities using Illumina sequencing of gene amplicons, it was found that *Ascomycota*, *Basidiomycota* and *Chlorophyta* dominated at the phylum level. And at the genus level, the dominant fungi were *Caloplaca*, *Trebouxia*, *Chaetomium*, *Clitopilus*, *Acanthostigma*, *Tolypocladium*, *Aspergillus*, *Toxicocladosporium* and so on. These biofilms influence changes in the mineral elements and lead to biodeterioration of the stone dog. For removing the biofilm from the stone dogs, through antimicrobial treatments and assessment, a mixture of siloxane and fluorosilane gave the best results, but one needs to be careful in applying antimicrobial treatments on stone monuments, as it may require a more comprehensive approach to examine the process.

Declarations

Disclosure of potential conflicts of interest

There are no potential conflicts of interest to declare.

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Tables

Table 1 Samples collected from the stone dogs

Samples number	Color	location
M1	Green	M173 stone dog's leg
M2	Green	M173 stone dog's back
M3	Orange	M177 stone dog's neck
M4	Grey and white	M177 stone dog's head
M5	Black	M177 stone dog's back
M6	Black	M0291 stone dog's back
M7	Black and green	M0202 stone dog's neck

Table 2 Antimicrobials

NO.	Material	Brushed area	
Material 1	BYS4002 BYB1007	5-1	fluorosilane and siloxane
Material 2	KY-104	5-2	biocide
Material 3	Sino-307	5-3	biocide
Control		5-4	

Table 3 Main elements of the stone dog

Component	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	CaO	MgO	Na ₂ O	TiO ₂	K ₂ O	P ₂ O ₅
(wt.%)	53.3	14.4	10.1	8.1	7.43	3.17	1.38	1.21	0.37
Component	MnO	SrO	SO ₃	ZnO	NiO	Cr ₂ O ₃	CuO	Cl	
(wt.%)	0.12	0.06	0.04	0.02	0.02	0.07	0.04	0.09	

Figures



Figure 1

Stone dogs A: Many stone dogs are in the yard; B,C,D,E, show the locations of different samples

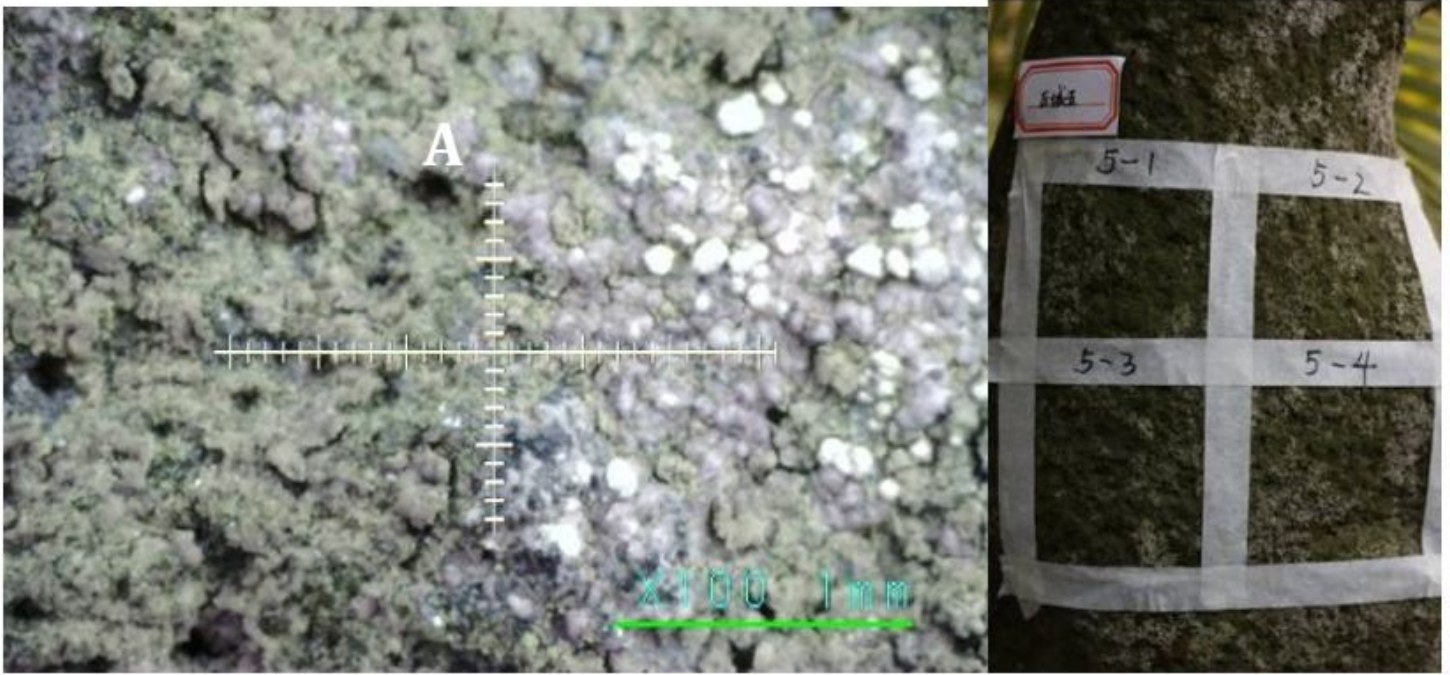


Figure 2

The sample was analysed by micromorphology using a portable microscope (A); and antimicrobial experiments (B).

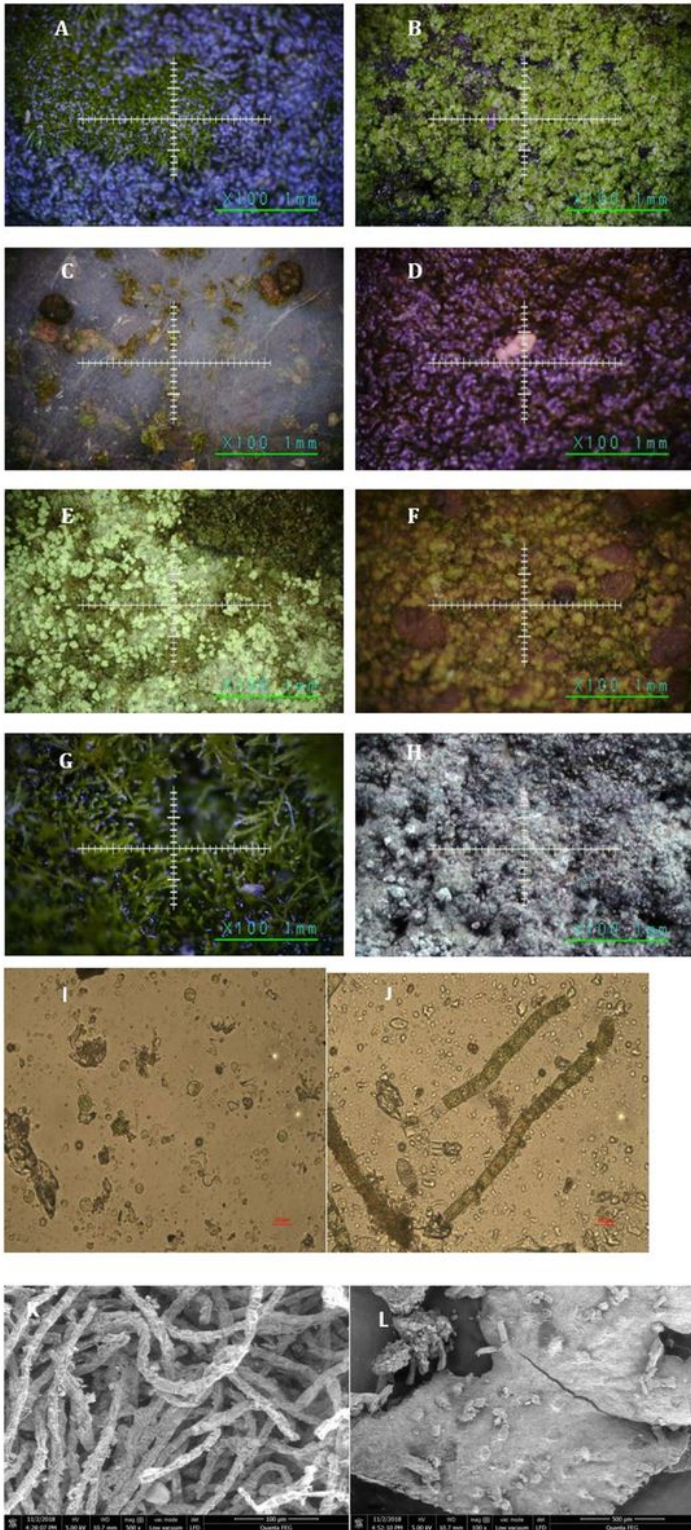


Figure 3

(A-H) Micromorphology of different colour biofilms on the surface of the stone dogs using a portable microscope; (I-J): Light micrograph of filamentous cyanobacteria, (I): single-celled and (J) filamentous; (K-L): Observation by SEM; (H): hyphae of the lichen; (L): hyphae attach themselves on the surface of the stone dog.

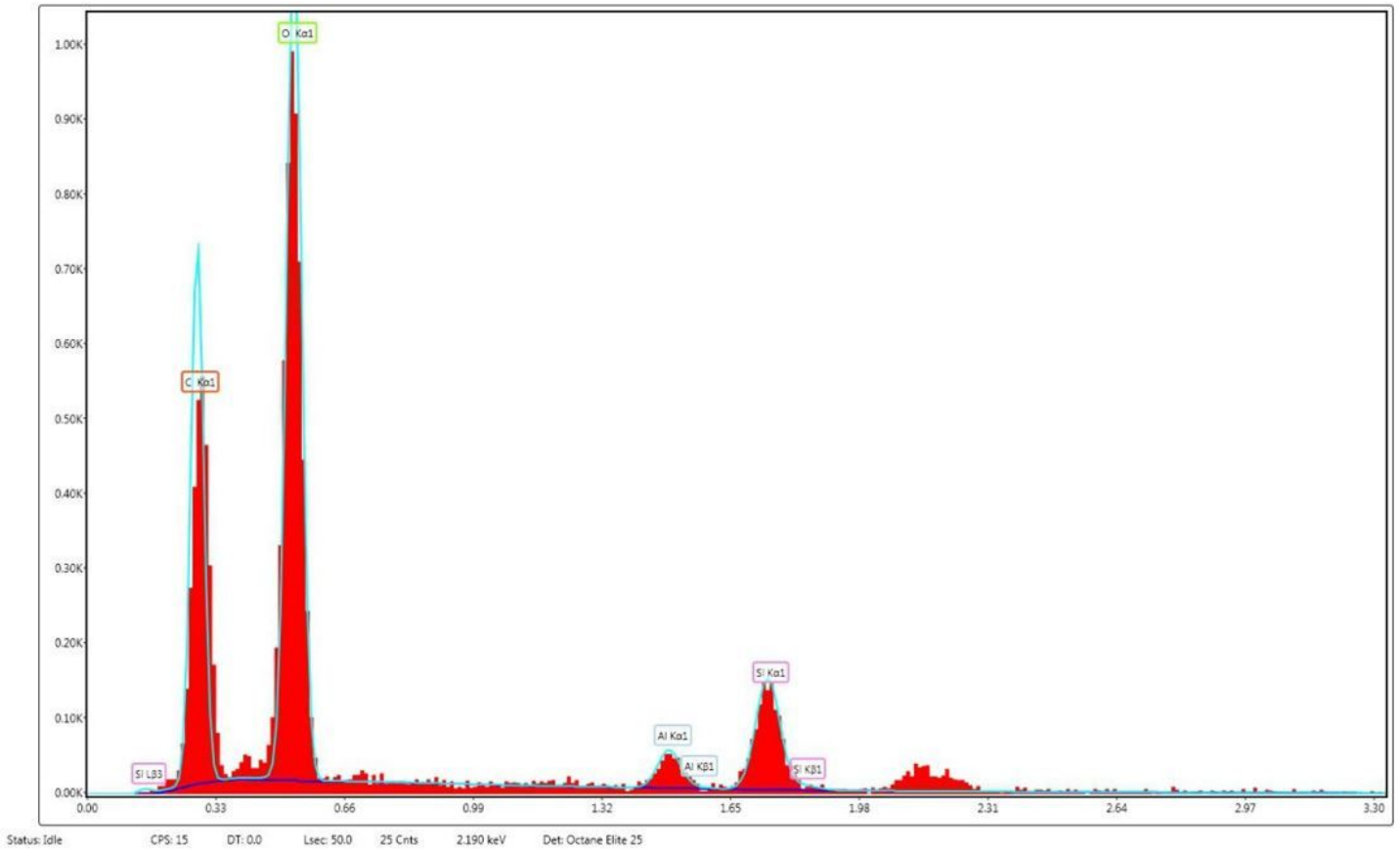


Figure 4

Element results of the biofilm by SEM-EDS analysis.

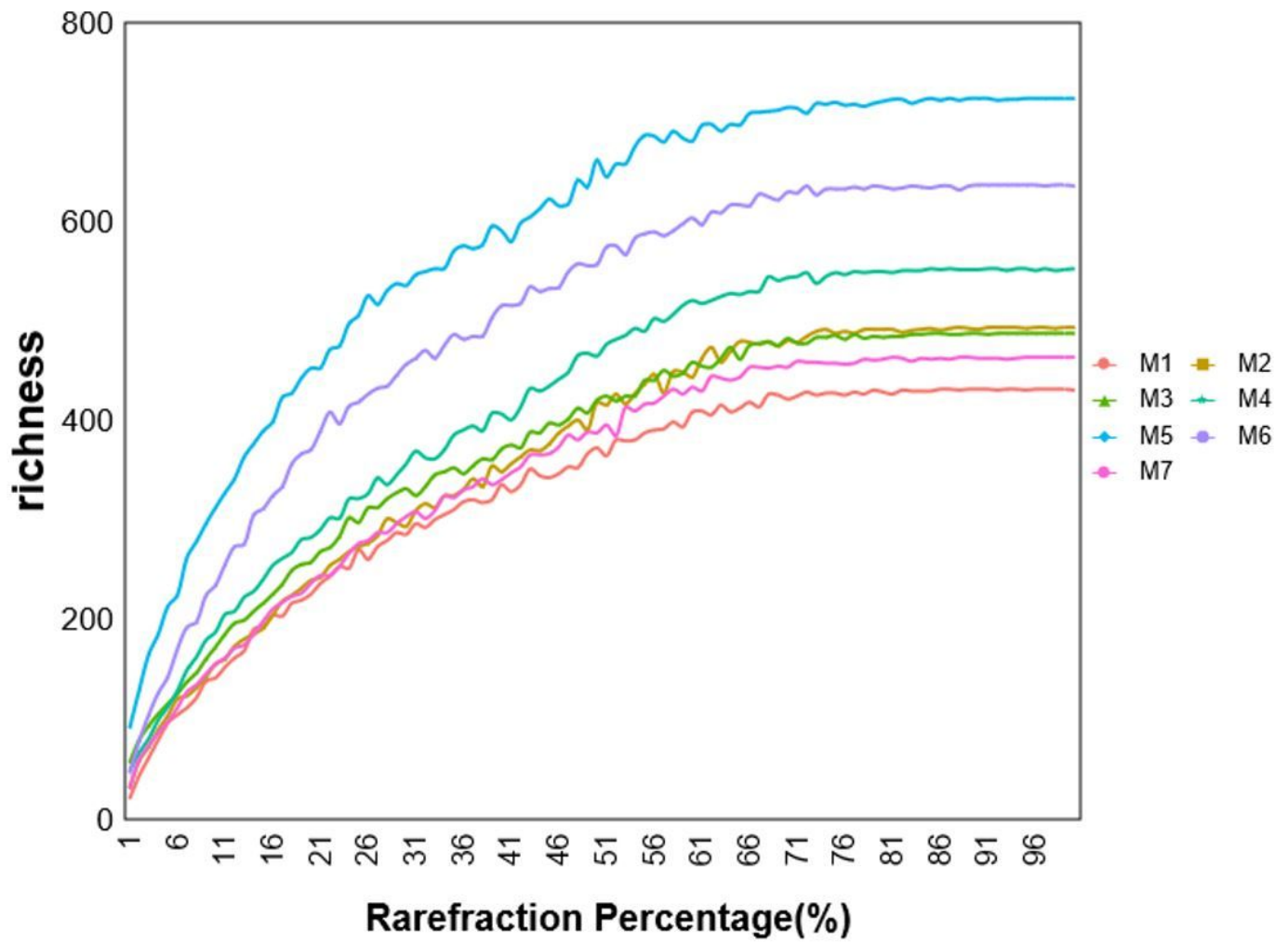


Figure 5

Rarefaction curves

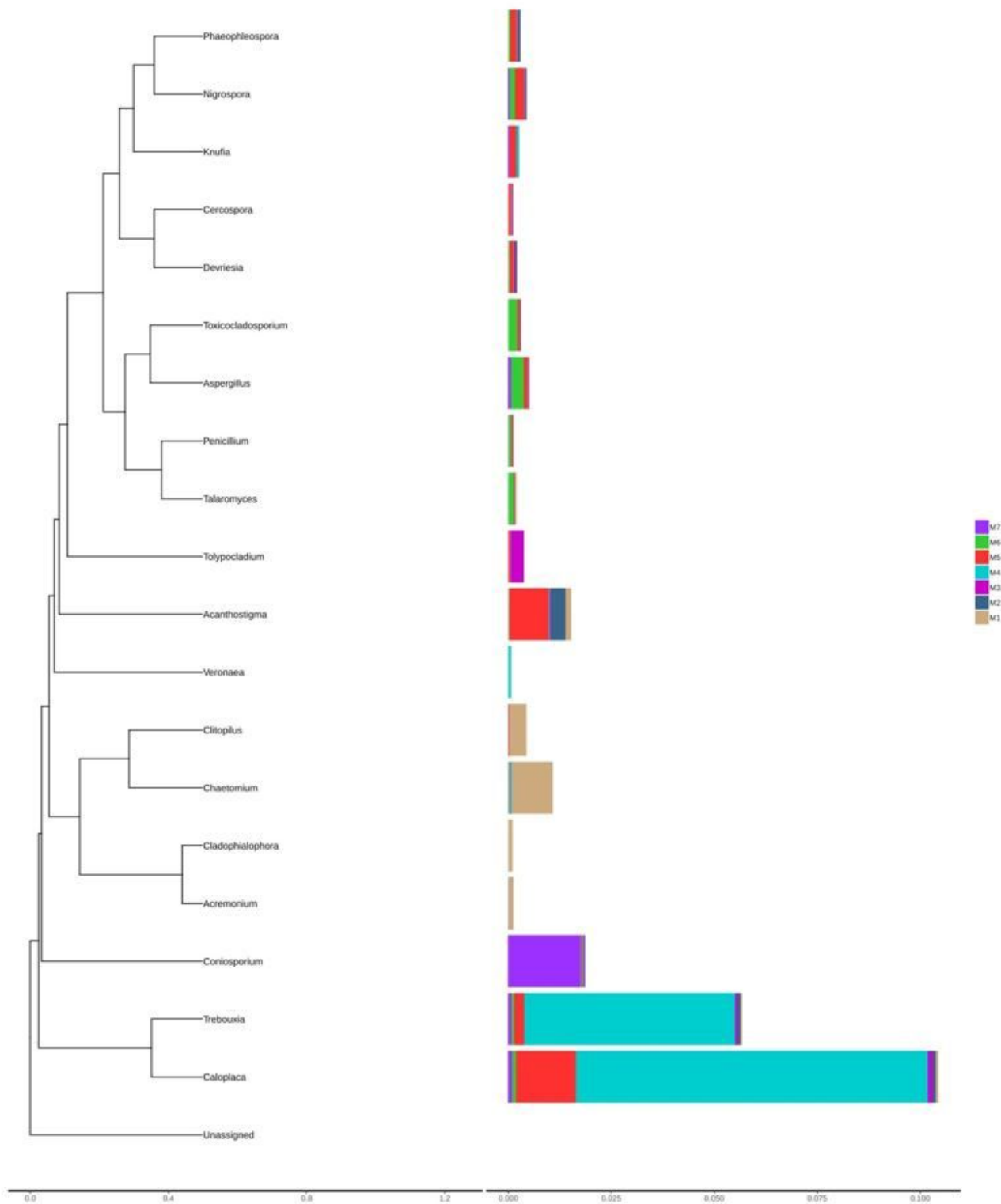


Figure 6

At the genus levels, the cluster tree was established by clustering analysis of samples under classification, and the horizontal bar chart of each sample under different water classification was displayed.

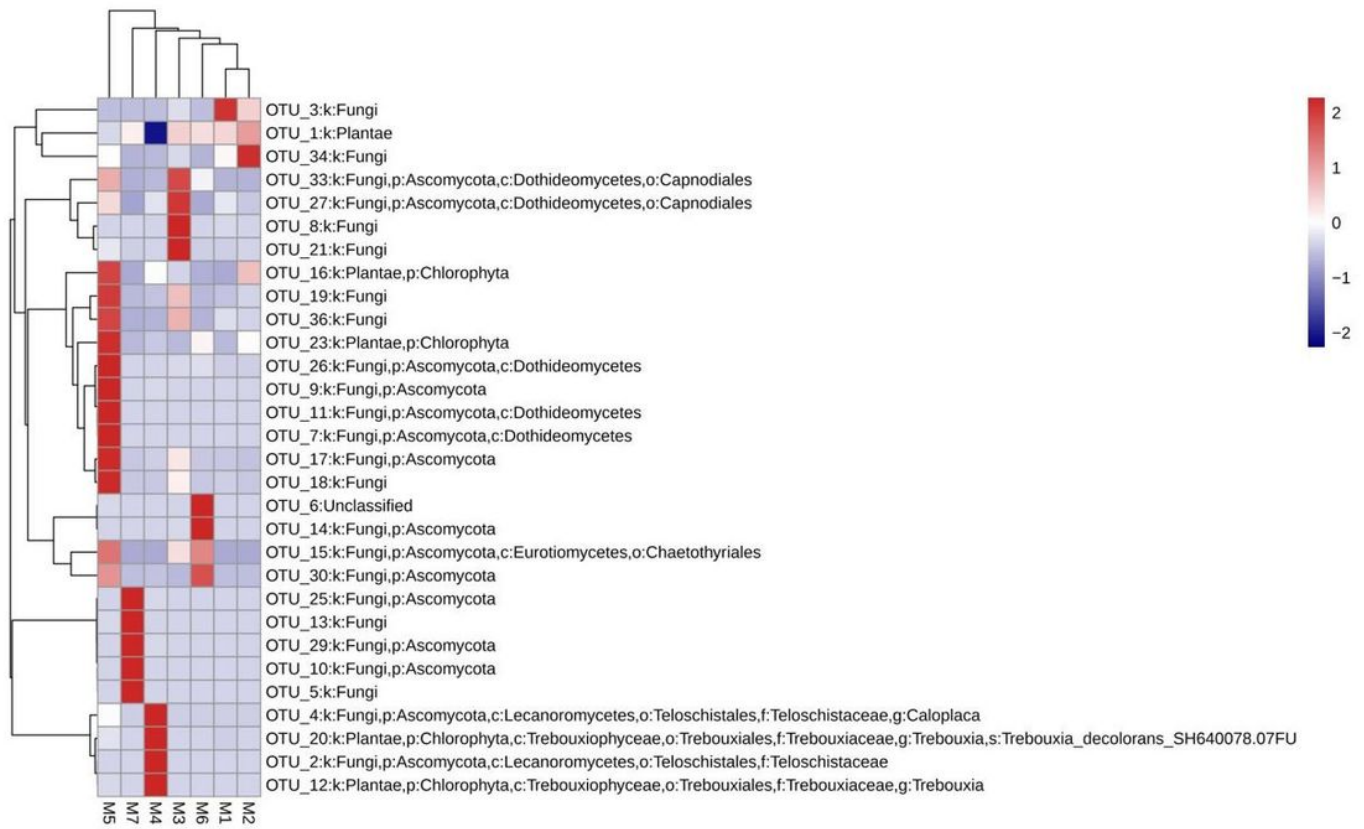


Figure 7

Cluster and heat map analysis of the biofilms communities from stone dogs

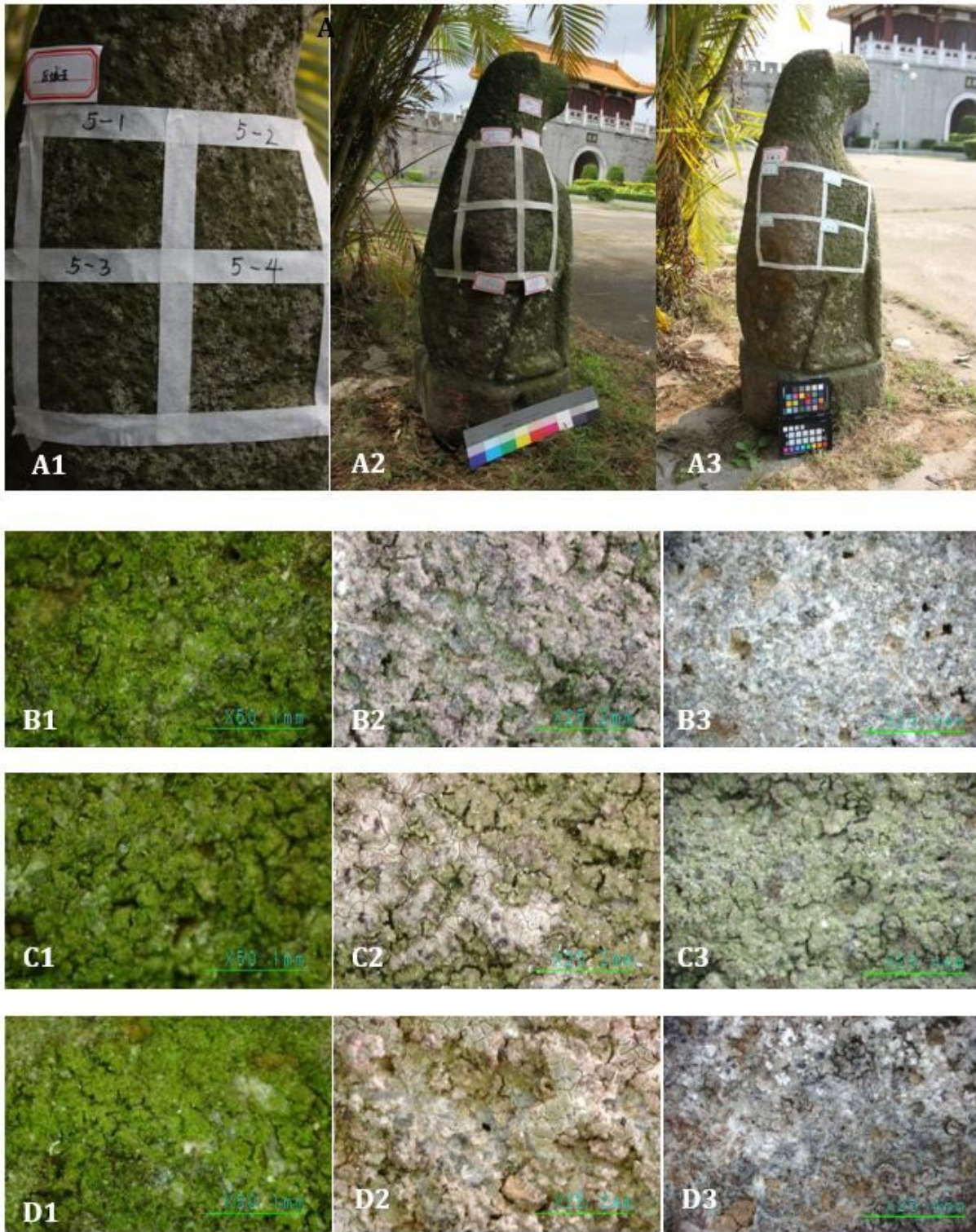


Figure 8

Comparison before and after antimicrobial treatments. A1: Before treatment; A2: 1 day after treatment; A3: 2 years after treatment; B1, B2, and B3 are respectively the micromorphology of 5-1 area before, 1 day after treatment, and 2 years after treatment; C1, C2, and C3 are respectively the micromorphology of 5-2 area before, 1 day after treatment, and 2 years after treatment; D1, D2, and D3 are respectively the micromorphology of 5-3 area before, 1 day after treatment, and 2 years after treatment.