Biodiversity's hidden treasure: biodeteriorated archaeological tombstones of Serbia

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In the present study, biological colonization on medieval tombstones from archaeological sites in Serbia has been investigated. Chemical analyses showed that the stone substrata were mostly of calcium carbonate, which is highly bioreceptive. Large areas of tombstones were covered with epilithic lichenized fungi and mosses, and microbiological analyses showed the presence of micromycetes and cyanobacteria. The dominant group of fungi recorded on tombstone surfaces was microcolonial fungi, now recognized as primary colonizers of stone substrata.

Keywords: Biodeterioration, cultural heritage, cyanobacteria, fungi, subaerial biofilms.

THE aim of the present study was to analyse the diversity of subaerial biofilm constituents on medievel tombstones from two archeological sites in Serbia. Deterioration of stone monuments is seen in European countries that are rich in cultural heritage. Stone materials have been extensively used throughout history for sculptures, monuments and in masonry as a means of artistic expression. The weathering of rocks, although an essential process for pedogenesis, represents an irreversible loss to civilization in case of stone monuments[1]. Memorial monuments are continuously affected by metereological elements such as temperature, humidity, rain, wind and condensation[2]. Biological colonization, along with physical and chemical factors plays a crucial role in the deterioration process[3]. Stone cultural heritage objects/monuments are particularly influenced by the colonization of different microorganisms (cyanobacteria, bacteria, micromycetes, algae) organized into subaerial biofilms (SABs), which occur on the interface between the stone surface and the atmosphere[4]. According to Prieto and Silva[5], stone bioreceptivity depends on its intrinsic characteristics such as composition and structure, while the intensity of microbial colonization is facilitated by ecological factors and atmosphere eutrophication. All stone materials are bioreceptive and therefore can be colonized to some extent[6]. All SAB-forming microorganisms are biodeteriogenic and are capable of causing deterioration of stone monuments, both mechanically and chemically. Biocorrosion of monuments is the result of chemical biodeterioration and is facilitated by the products of microbial metabolism such as enzymes, chelating agents, acids and extracellular polymeric substances[3]. SAB-forming micromycetes and phototrophs penetrate stone materials causing mechanical damage[7]. In addition, SABs also cause aesthetic alterations of stone monuments, due to production of pigments by various microorganisms, as protective compounds against environmental stress factors, especially UV-radiation[8]. Hence, production of pigments and other colouring agents leads to chromatic variations of the stone surface and significantly decreases the aesthetic value of the monuments. It is now a global problem in cultural heritage conservation[9].

In the later stages of biological succession of stone colonization, more advanced communities consisting of lichens, mosses and vascular plants can occur. Lichens develop on stone surfaces after they have been partially enriched with air deposits and previous colonizers[10]. Bryophyte communities can develop on stone surfaces after protosoil formation, which is favoured by previous colonization and air-borne particles retained on SAB, or accumulated in crevices or holes[11].

Material and methods

Site description

Samples were taken from medieval tombstones belonging to two archeological sites, Mramorje in Perućac and Rastište in southwestern Serbia. The archeological site Mramorje in Perućac (43°57′14″N, 19°25′16″E, altitude: 239 m amsl) is located on the bank of the River Drina, between the road and river, and is the best preserved necropolis in the region with 100 tombstones. The archeological site in Rastište (43°56′20″N 19°19′32″E, altitude 520 m amsl) belongs to the Tara National Park first protection zone and is surrounded by forests and fields. The necropolis on this archeological site consists of 38 tombstones.

The tombstones classified as stećaks have been recorded in all the areas along the present border municipalities between Serbia, Bosnia and Herzegovina, and

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Montenegro, as well as in the neighbouring areas in western and southwestern Serbia. Burial under these stones was conducted in the 14th and 15th centuries in Serbia. The individual inscriptions on the stones testify to this practice. The tombstones in both necropoleis are arranged in regular rows and have an east–west orientation. Both archeological sites are protected by the Republic of Serbia as Monuments of Culture of Exceptional Importance. In 2009, they were nominated for the UNESCO World Heritage List. As of July 2016, Stećci Medieval Tombstones Graveyards have been included in the UNESCO World Heritage List.

Chemical sampling and analyses

The sampling for chemical analyses was done by taking small stone fragments from the tombstone surface with visible alterations. Samples were embedded in cold polymerized resin (methyl-metaacrylate) and polished with sandpaper, ultra fine (P2000). Cross-sections were used for light microscopy analyses in transmitted light and also for SEM-EDS analyses. Cross-sections were observed under a metallographic microscope (Reichert Wien Universal MeF) with magnification of objective 20×. Electron images were obtained and electron microprobe analyses were done at the University of Belgrade, Faculty of Mining and Geology, using a scanning electron microscope (SEM; JEOL JSM–6610LV (JEOL Ltd, Tokyo, Japan); coupled to an energy dispersive X-ray spectrometer (Oxford Instruments X-Max). Samples were gold-coated (d = 15 nm, density of gold ρ = 19.2 g/cm^3) using a sputter coater (Leica EM SCD005, Wetzlar, Germany). A W-filament electron gun with an acceleration voltage of 20 kV was used to obtain X-ray spectra, secondary electron and backscattered electron images.

Microbiological sampling and analyses

For SAB analyses, samples from the surfaces of the monuments were taken in spring 2011. The non-aggressive adhesive tape method was used for sampling phototrophic microorganisms and micromycetes for direct observation of SAB[12]. The non-destructive adhesive tape method was carried out by gently applying a strip of adhesive tape to the tombstone surface being studied, which was then removed to sterile glass microscope slides and kept in sterile conditions prior to laboratory processing. Small pieces of strips were then sorted for microscopic analyses. After rehydration in a modified Knöpp medium, the samples were analysed by stereomicroscope (Stemi DV4, Zeiss) and a optical microscope (Zeiss Axio-ImagerM.1, with software AxioVision Release 4.6).

Sterile cotton swabs method was used for sampling of culturable micromycetes. Collected samples were diluted in sterile distilled water (10 ml) and shaken vigorously within 10 min. Malt extract agar (MEA), with antibiotic streptomycin added, was inoculated with final suspension (1 ml). Every sample was processed three times. Inoculated MEA was then incubated using a thermostat (25°C). Axenic cultures of each isolate were obtained through single spore transfer of primary isolates onto standard mycological media: MEA, potatodextrose agar and Czapek agar. All the cultures were maintained for 7 days at room temperature, and their macroscopic and microscopic features were investigated.

Identification of biodeteriogens

The detected cyanobacteria and algae were identified according to morphological characteristics using identification keys by Komàrek *et al.*[13,14], Komàrek[15] and Laundon[16].

Identification of micromycetes was based on the macroscopic features of the colonies growing on selected nutrient media and the micromorphological characteristics of the conidiogenous apparatus using identification keys by Raper and Fennell[17], Pitt[18], Ellis and Ellis[19], and Samson *et al.*[20].

Lichens and mosses present on the surfaces of tombstones were identified during *in situ* observation using dichotomous keys by Smith *et al.*[21] and Frey *et al.*[22].

Results and discussion

Chemical analyses

Chemical analyses (SEM-EDS) showed the presence of calcium, carbon and oxygen in the spectrum from the examined stone samples of tombstones with thalli of lichens from both archeological sites. This suggests that the examined stone substrata were mostly of calcium carbonate (Figure 1). The chemical composition of stone substratum is a factor influencing the bioreceptivity of the stone[6]. Calcareous stones are highly susceptible to microbial colonization due to optimal pH and porosity[23]. Overgrowth of phototrophic microorganisms in Roman hypogea (Italy) was facilitated by high calcareous stone porosity[24]. Miller *et al.*[1] demonstrated high degree of calcareous substrata bioreceptivity *in vitro*.

In situ observations

The examined monuments showed clear signs of biodeterioration, such as changed stone texture, large areas with chromatic variations and the presence of macrofouling in the form of epilithic lichens and mosses (approx. coverage 95%). On stone monuments from Mramorje, large areas of the upper surface of the stećaks were covered

with black crust of predominant cyanobacteria *Nostoc commune*. Crustose thalli of lichens *Aspicilia calcarea* and *Verrucaria* sp., and placodioid thalli of *Lobothallia radiosa* were abundant on the surface of all monuments, but predominantly on the lateral sides. *Gymnostomum* sp. and *Syntrichia ruralis* were the only moss species documented in the form of scattered tufts on the surface of all monuments, but predominantly on the north lateral sides. Large areas of the monuments (approx. coverage >80%) at Rastište were covered with thalli of the foliose lichen *Collema auriforme* together with three moss species emerging from the cracks of stone surfaces: *Hypnum cupressiforme*, *Homalothecium sericeum* and *Schistidium apocarpum* (Table 1 and Figure 2).

Lichen colonization has great ecological importance due to their pedogenetic activity on the stone substrata[25]. Lichens deteriorate the stone surface chemically and mechanically. Carbon dioxide, as a product of lichen respiration, is transformed into carbonic acid within the thallus. In addition to carbonic acid, other products of lichen metabolism, especially corrosive organic acids can form cation–ligand complexes with minerals present in the stone substrata. Lichen metabolites lead to changes in stone composition in close proximity of lichen thalli via depletion of the main chemical elements and their accumulation, inside the thallus especially Ca^{2+} (ref. 26). As a result of hyphal deep penetration and periodic thallus contraction and swelling, lichens cause physical damage[27]. With their slow and constant growth they can have high coverage on the stone surfaces with undesired chromatic alterations. The metallographic microscope in reflected light mode, at 320× magnification identified a lichen layer of about 60 µm thickness, as well as the layer beneath, which had degraded, and structural changes approximately of 80 µm were documented (Figure 3).

Development of mosses is less rapid, especially on humus-free stones. The principal source of humus deposits is the accumulation of dead microbial cells and atmospheric particles. The chemical and mechanical activity of these organisms on stone monuments cannot be imperceptible, even though they have only rhizoids in direct contact with the stone surface and not the true root systems. However, the moss biodeterioration capacity is due to accumulation of Ca^{2+} ions[28,29]. Also, bryophytes are indicators of humid conditions, since they require high moisture to complete their life cycle.

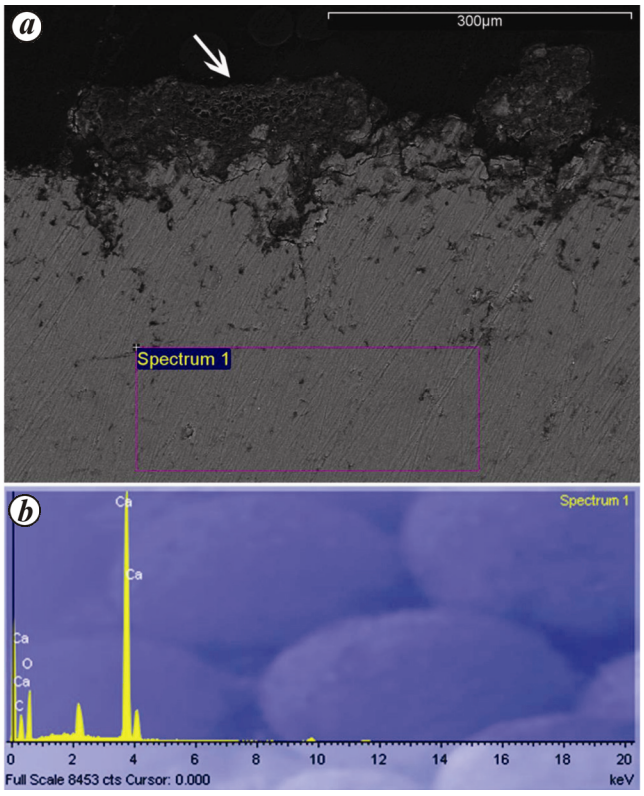

Figure 1. SEM-EDS analyses of stone fragment. ***a***, SEM image of cross-section; arrowhead points to the epilithic lichen thallus. ***b***, EDS spectrum.

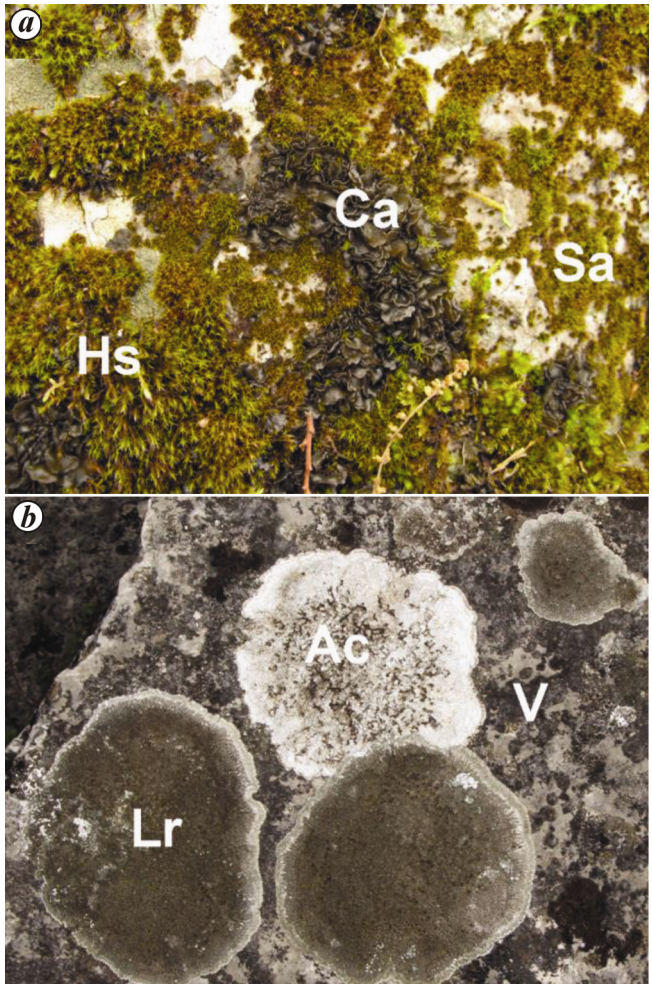

Figure 2. Lichens and mosses. ***a***, *Homalothecium sericeum* (Hs), *Collema auriforme* (Ca) and *Schistidium apocarpum* (Sa); ***b***, *Lobothallia radiosa* (Lr), *Aspicilia calcarea* (Ac) and *Verrucaria* sp. (V).

Table 1. Diversity of biodeteriogens on stone surfaces

	Mramorje	Rastište
Cyanobacteria	*Gloeocapsa compacta* Kützing *Gloeocapsa sanguinea* (C. Agardh) Kützing *Gloeocapsopsis* sp. Geitler ex Komárek *Gloeothece* sp. Nägeli *Gloeothece incerta* Skuja *Gloeocapsopsis pleurocapsoides* (Novácek) J. Komárek and K. Anagnostidis *Nostoc commune* Vaucher ex Bornet and Flahault *Scytonema myochrous* (Dillwyn) C. Agardh ex Bornet and Flahault	*Gloeocapsa compacta* Kützing *Gloeocapsa violacea* Kützing *Gloeocapsopsis pleurocapsoides* (Novácek) J. Komárek and K. Anagnostidis *Gloeothece* sp. Nägeli *Nostoc commune* Vaucher ex Bornet and Flahault *Scytonema myochrous* (Dillwyn) C. Agardh ex Bornet and Flahault *Tolypothrix byssoidea* (C. Agardh) Kirchner
Algae		*Desmococcus olivaceus* (Persoon ex Acharius) J. R. Laundon
Actinomycetes	*Nocardia* Trevis sp.	
Fungi	*Alternaria* Nees spp. *Aspergillus* P. Micheli ex Link sp. *Cladosporium cladosporioides* (Fresen.) G.A. de Vries *Drechslera dematoidea* (Bubák and Wróbl.) Scharif *Epicoccum purpurascens* Ehrenb *Fusarium* Link sp. MCF *Mycelia sterilia* *Paecylomyces varioti* Bainier *Phoma* Fr. sp.	MCF *Mycelia sterilia* *Phoma* Fr. sp. Yeasts
Lichens	*Aspicilia calcarea* (L.)Körb *Lobothallia radiosa* (Hoffm). Hafellner *Verrucaria* Scop. sp.	*Collema auriforme* (with.) Coppins & J.R. Laundon
Mosses	*Gymnostomum* Hedw sp. *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr	*Hypnum cupressiforme* Hedw. *Homalothecium sericeum* (Hedw.) Schimp. *Schistidium apocarpum* (Hedw.) Bruch & Schimp.

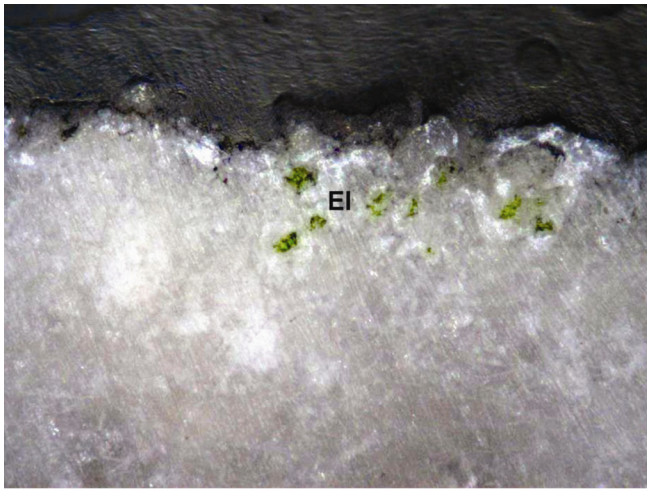

Figure 3. Cross-section of tombstone surface fragment (metallographic microscope, 320×). El, Endolithic lichen talli.

Microscopic observations

Microscopy (light and SEM) is the most suitable tool for analysing SAB and bioderioration process[12]. Optical microscopy showed the presence of SAB-forming photoautotrophs and filamentous fungi. On the surfaces of tombstones from Mramorje, eight taxa of cyanobacteria were detected: *Gloecapsa compacta*, *G. sanguinea*, *Gloeocapsopsis* sp., *G. pleurocapsoides*, *Gloeothece incerta*, *Gloeothece* sp., *Nostoc commune* (predominant taxa) and *Scytonema myochrous* (subdominant). Cells of green algae were not documented during light microscopy of adhesive tape samples. On the tombstone surfaces from Rastište, seven taxa of cyanobacteria were recorded: *G. compacta*, *G. violacea*, *G. pleurocapsoides*, *Gloeothece* sp., *N. commune* (predominant), *S. myochrous* and *Tolypothrix byssoidea*. *Desmococcus olivaceus* was the only green alga recorded on any of the examined surfaces. The microfouling community consisted predominantly of cyanobacteria in dense clusters with thick polysaccharide sheaths formed due to extreme environmental conditions (Table 1 and Figure 4).

Cyanobacteria and algae are considered as the pioneering colonizers of stones. Adaptations of stone-dwelling algae and cyanobacteria include the formation of polysaccharide sheaths that allow fast absorption as well as slow humidity discharge. This sheath eases cell/surface

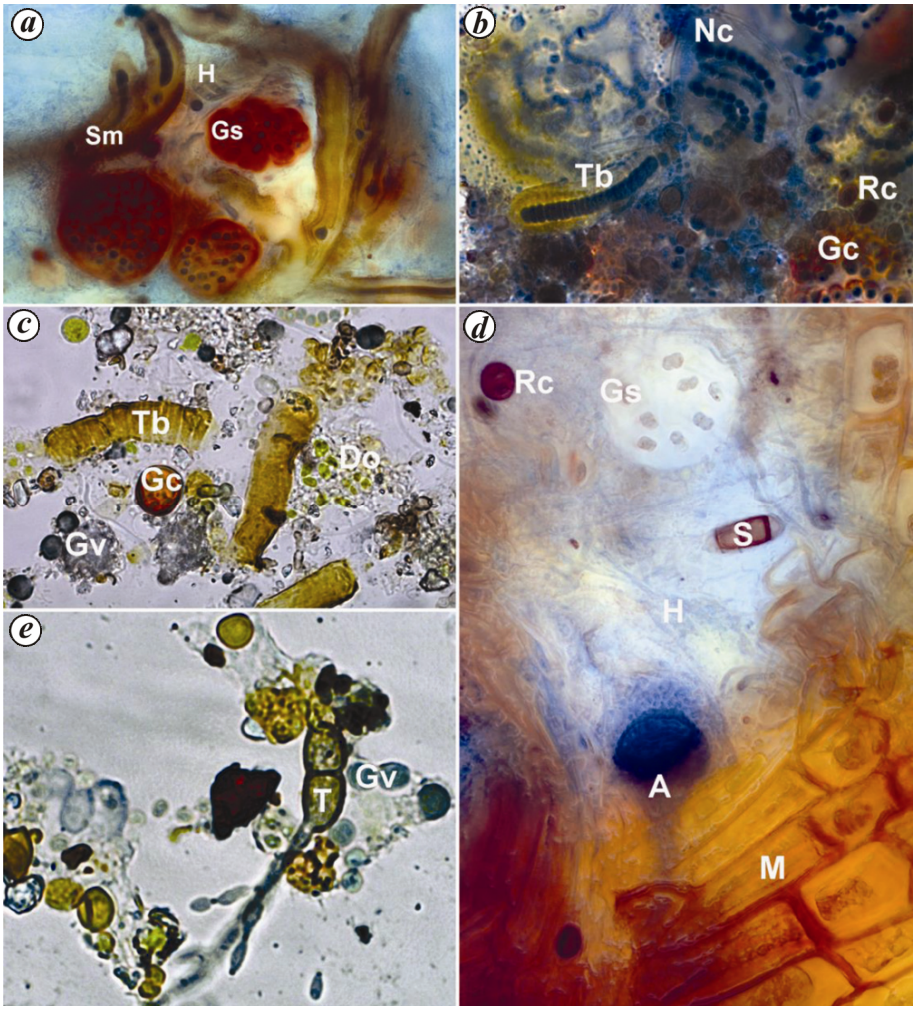

Figure 4. Microorganisms on tombstone surface. ***a***, *Scytonema myochrous* (Sm), *Gloeocapsa sanguine* (Gs), Hyphae (H); ***b***, *Nostoc commune* (Nc), Resting cells (Rc), *Tolypothrix byssoidea* (Tb), *Gloeocapsa compacta* (Gc); ***c***, *Tolypothrix byssoidea* (Tb), *Gloeocapsa compacta* (Gc); *loeocapsa violascea* (Gv), *Desmococcus olivaceus* (Do); ***d***, Hyphae (H), Fungal sporae (S), *Aspergillus* sp. (A), G*loeocapsa sanguine* (Gs) – Young colony; Moss cells (M) and resting cells (Rc); ***e***, Germinating teliospore (T) and *Gloeocapsa violascea* (Gv).

adhesion and to overcome prolonged arid periods[30]. Phototrophic microorganisms (cyanobacteria, green microalgae and lichens) can develop on stone surfaces even in the absence of organic matter, and are of particular importance as pioneer organisms in the colonization of stone materials. Three main defence strategies were developed by cyanobacteria for protection against damage caused by UV-radiation: migration, repair mechanisms, and production of sunscreen pigments (scytonemin, gleocapsin, mycosporine-like aminoacids and carotenoids)[31]. *Nostoc* species also produce detoxifying enzymes like superoxide dismutase and catalase which prevent and repair UV-radiation-induced damages. Approximately 10% and 15% dry weight of a colony is made up of mycosporine-like amino acids and scytonemin respectively[32]. Due to the fact that organisms such as lichens and cyanobacteria are poikilohydric, their growth is not limited by unfavourable xeric periods, although a better bioclimate favours their colonization[33]. These phototrophs may be predominant on the vertical surfaces of monuments or other cultural heritage assets. In general, they possess survival mechanisms to resist unfavourable environment due to their highly developed outer envelopes rich in sunscreen pigments. According to Danin and Caneva[34], chasmoendolithic cyanobacteria degrade calcareous substrata utilizing pressure within the stone via water uptake, cell-mass expansion, and precipitation of oxalates and carbonates around the cells. Precipitation of calcium salts on the surface of cyanobacteria colonizing calcareous substrata suggests Ca^{2+} migration from adjoining sites. Photosynthetic microorganisms deposit $CaCO_3$ during daylight and solubilize it again at night as a result of changes in carbonate concentration. Ca^{2+} mobilization as well as trapping of released calcite particles in the mucilaginous cyanobacterial sheaths lead to the degradation of calcareous substrata[35].

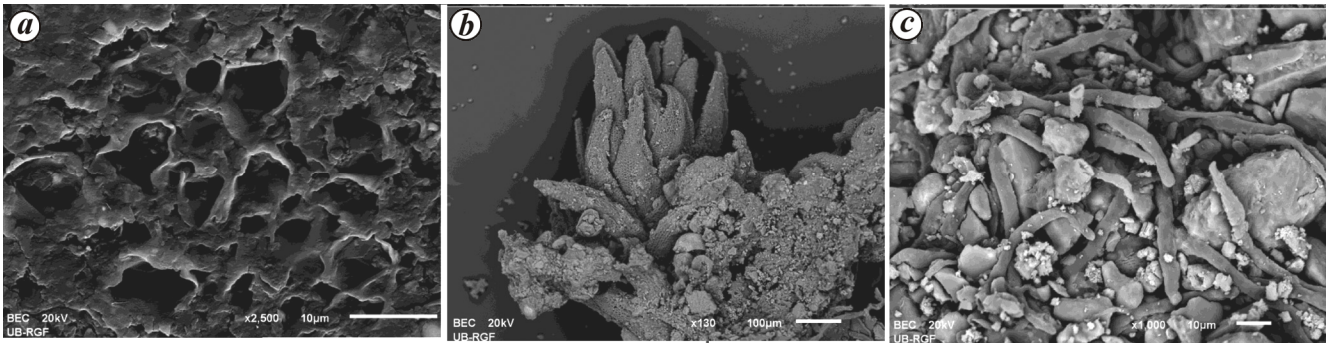

Figure 5. SEM images of biodeteriorated tombstone surface: ***a***, lichen; ***b***, moss; ***c***, filamentous cyanobacteria.

On the tombstone surfaces from Mramorje, fungi belonging to the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Drechslera*, *Epicoccum*, *Paecylomyces* and *Phoma* were documented (Table 1). The fungal community recorded on tombstones on this archeological site was dominated by melanized microfungi, dematiaceous hyphomycetes, with the highest isolation frequency (100%). Yeasts were also observed in all primary isolates and were abundant (isolation frequency >75%). During the cultivation of primary isolates from both sites, unidentified rock-inhabiting fungi[36] or micro-colonial fungi, producing slowly expanding, hairy colonies with a central protuberance (umbonate), were recorded in all samples (100%). These fungi are now recognized as primary colonizers of stone substrata[37]. The ability to grow meristematically ensures the colonies an optimal surface/volume ratio and is hypothesized to enhance the ability to survive under adverse conditions such as extreme temperature, low water availability, unfavourable acidity, nutrient deficiency and high UV-exposure[38,39]. Free-living micromycetes, along with lichen mycobionts, were the most numerous organisms documented on the analysed monuments. Micromycetes have ecological advantages over stone-colonizing bacteria and algae due to their higher tolerance to low water potential, their efficient modes of reproduction, and their ability to grow and proliferate even if nutrient concentration in the environment is low[40]. Another significant advantage of micromycetes is their capacity to efficiently translocate nutrients through hyphal network, enabling them to explore and colonize heterogeneous environments[41].

The main SAB constituents detected using light microscopy were confirmed by SEM (Figure 5). SABs forming phototrophs and heterotrophs develop concomitantly and thus cause synergistic biodeterioration effect. Organic matter produced by phototrophs has been utilized by bacteria and fungi, which consequently excrete organic acids that can solubilize substratum mineral components. Continuous iteration of microbial attacks further weakens the mineral matrix through drying and wetting cycles, and following expansion and contraction[23].

Conclusion

In the present study we have analysed the biodeterioration process on stone monuments. Results of the study help in developing and maintenance procedures, treatment and safeguarding of tombstones within the proposed Conservation and Restoration Programme in Serbia. The study of organisms involved in the biodeterioration process and characterization of the changes in stone structure caused by biodeteriogenic agents could establish novel criteria for eradication of biodeteriogens.

Collaboration among biologists, chemists, conservators and other specialists is necessary to successfully protect stone cultural heritage monuments. Monitoring of biofilm formation is of utmost importance not only for stone cultural heritage objects/monuments, but also for the prevention of weathering on modern building exteriors.

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