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## BIOFILM FORMING MICROORGANISMS ON VARIOUS SUBSTRATA FROM GREENHOUSE OF BOTANICAL GARDEN “JEVREMOVAC”

**ABSTRACT:** Diversity of subaerial biofilm forming cyanobacteria, algae and fungi was investigated on 10 different substrata from greenhouse of Botanical Garden “Jevremovac”. Out of 37 documented taxa, 16 cyanobacterial and 10 algal taxa were identified. Remaining 11 taxa belong to the Kingdom of Fungi. The highest diversity of biofilm forming microorganisms, a total of 24 taxa, was detected on the corroded metal surface, while significantly lower number of taxa was recorded on other examined substrata. Cyanobacterium *Porphyrosiphon* sp., diatom *Achnanthes* sp. and green algae *Chlorella* sp. and *Chlorococcum minutum* were the most frequently encountered photosynthetic components of biofilms. In all analyzed samples, *Trichoderma* sp., followed by *Cladosporium* sp. and *Rhizopus stolonifer*, were the most frequently identified fungi.

**KEYWORDS:** algae, biofilm, cyanobacteria, fungi, greenhouse

### INTRODUCTION

In natural conditions, phototrophic and heterotrophic microorganisms are able to colonize and subsequently form ubiquitous, self-sufficient, miniature microbial ecosystems on all substrata where direct contact with the atmosphere and solar radiation occurs (Gorbushina, 2007). Process of establishing these complex microbial communities, known as subaerial biofilms (SABs), depends on

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substratum bioreceptivity, biology of colonizing microorganisms and wide range of environmental factors such as temperature, humidity, pH, solar radiation, water and nutrient availability (Gu and Mitchell, 2006; Macedo *et al.*, 2009). In early stages of SAB formation, cyanobacteria and algae use CO<sub>2</sub> from the atmosphere and sunlight as their carbon and energy source and produce metabolites which serve as nutrient source for incoming heterotrophic bacteria and fungi (Ljaljević Grbić *et al.*, 2010). In addition, various air-borne organic and inorganic deposits and animal remains help fill nutrient requirements for heterotrophic SAB forming microorganisms (Suihko *et al.*, 2007). Although most biofilms only contain complex consortia of algae, cyanobacteria, heterotrophic bacteria, fungi and protozoa, in advance stages of the colonisation, more complex organisms such as lichens, mosses and vascular plants can occur (Ljaljević Grbić *et al.*, 2009; Stupar *et al.*, 2014).

Changes in the structure and appearance of the substratum occur as a result of biofilm development. Discoloration, due to pigment excretion, depends on specific physiology of the SAB involved species and is influenced by changes in physiological state of the cells and the environmental conditions (Cappitelli *et al.*, 2008; Warscheid and Braams, 2000). Additionally, SAB forming microorganisms secrete various extracellular polymeric substances (EPS) to maintain moisture levels, enable mutual binding of microbial cells and adhesion to the substratum (Warscheid and Braams, 2000; Macedo *et al.*, 2009). However, EPS may potentially cause alteration of physico-chemical properties of the substrata due to retained water (Brehm *et al.*, 2005; Keshari and Adhikary, 2013). Fungi produced organic acids and enzymes interact with released CO<sub>2</sub> resulting in pH change of the substratum, which further facilitates the mechanical degradation (Gorbushina *et al.*, 2007). Moreover, chemical reactions between organic acids and minerals ensue bio-weathering and formation of secondary minerals on the attacked substrata.

The aim of this research was to study the diversity of SAB forming microorganisms on different substrata from greenhouse of Botanical Garden “Jevremovac”.

## MATERIALS AND METHODS

Sampling was done in 2010 from different substrata within the greenhouse of Botanical Garden “Jevremovac”, University of Belgrade, Faculty of Biology, Institute of Botany.

### *Sampling site*

Sampling of SAB forming cyanobacteria, algae and fungi was conducted on various substrata from the greenhouse of Botanical Garden “Jevremovac”. The Botanical Garden was founded in 1874 by the decree of the Ministry of Education of the Kingdom of Serbia, at the suggestion of famous Serbian botanist Josif Pančić. The greenhouse, from which samples were taken, was built in 1892 and covers the area of 500 m<sup>2</sup>. Since the time of its construction, numerous tropical, sub-tropical

and desert plants have been grown in two wings connected by a central dome. From 1892 to 2010 no work was done on the reconstruction of the greenhouse. Today, due to its exceptional architectural value, it is protected by the law.

### *Sampling*

Samples for algological and mycological analyses were collected from the surfaces of 10 different substrata with visible SAB formation: wood (Wd), stone (St), sand (Sd), clay (Cl), mortar (Mr), concrete (Co), metal (Mt), nylon (Ny), putty (Pt), and glass (Gl) (Figure 1).



*Figure 1.* Examined substrata, with visible alterations, from the greenhouse of Botanical Garden “Jevremovac”: a. wood; b. stone; c. mortar; d. clay; e. sand; f. concrete; g. putty; h. metal; i. glass; j. nylon.

### *Algological analyses*

Algological analyses were conducted on samples acquired using two methods: scraping and non-aggressive adhesive tape sampling (Gaylarde and Gaylarde, 1998). After rehydration in modified Knöps medium, samples were analyzed using stereomicroscope (Zeiss Stemi DV4) and a light microscope (Zeiss Axio-Imager M1, with software AxioVision Release 4.6). The observed cyanobacteria and algae were identified to species or genus level, on the base of cellular morphology, using appropriate literature (Starmach, 1972; Krammer and Lange-Bertalot, 1988; Komarek and Anagnostidis, 1998; Komarek and Anagnostidis, 2005).

### *Mycological analyses*

Sampling for the mycological analyses was done using sterile cotton swabs and adhesive tape method. Sterile swab samples were diluted in 10 mL sterile distilled water and shaken mechanically for 10 min, after which 1 mL of the resulting suspensions was inoculated on malt extract agar (MEA) medium with 500 mg streptomycin per liter (Booth, 1971). The inoculated plates were incubated in a thermostat at  $25 \pm 2$  °C. After incubation period of 7 days, pure fungal cultures were obtained by re-isolation of primary isolates onto the selective nutrient media: MEA, potato dextrose agar (PDA), and Czapek Dox agar (CzA). Re-isolated cultures were incubated 7 days at  $25 \pm 2$  °C. Isolated fungi were identified to species or genus level, based on the macroscopic features of colonies and the micro-morphology of the reproductive structures, using the appropriate identification keys (Ainsworth *et al.*, 1973; Von Arx, 1974; Ellis, 1971; Ellis and Ellis, 1997; Samson *et al.*, 2004).

To confirm the existence of fungal growth and identify the type of fungi present at the sampling points, the non-aggressive adhesive tape sampling method was used (Urzi and de Leo, 2001). Samples were collected by pulling the adhesive tape off the surface of substrata with a slow and steady force, after which they were stained with Lactophenol Cotton Blue and put on slides for light microscopy.

## RESULTS AND DISCUSSION

A total of 37 biofilm forming cyanobacteria, algae and fungi was identified on the surfaces of 10 substrata examined from greenhouse of Botanical Garden “Jevremovac“. All identified taxa are presented in Table 1. The highest microbial diversity, a total of 24 taxa, was detected on the corroded metal surface. In contrast, significantly lower number of taxa was identified as SAB forming microorganisms on other examined substrata.

Table 1. Identified taxa on various substrata from greenhouse

Identified taxa		Substrata									
		Wd	St	Sd	Cl	Mr	Co	Mt	Ny	Pt	Gl
Cyanobacteria	<i>Aphanothece pallida</i> (Kützing) Rabenhorst							+			
	<i>Chondrocystis dermochroa</i> (Nägeli) Komárek & Anagnostidis							+			
	<i>Chroococcus lithophilus</i> Ercegovic							+	+		
	<i>Chroococcus varius</i> A. Braun				+			+			
	<i>Chroococcus</i> Nägeli sp.		+								
	<i>Gloeocapsa atrata</i> Kützing							+			
	<i>Gloeocapsa novacekii</i> Komárek & Anagnostidis							+			
	<i>Gloeocapsa</i> Kützing spp.							+	+		
	<i>Gloeocapsopsis crepidinium</i> (Thuret) Geitler ex Komárek						+				+
	<i>Gloeocapsopsis</i> Geitler ex Komárek sp.							+			
	<i>Lyngbya truncicola</i> Ghose			+							
	<i>Nostoc</i> Vaucher ex Bornet & Flahault sp.						+		+		
	<i>Phormidium</i> Kützing ex Gomont sp.		+					+			
	<i>Porphyrosiphon</i> Kützing ex Gomont sp.		+		+			+			
	<i>Pseudocapsa dubia</i> Ercegovic							+			
<i>Synechococcus elongatus</i> (Nägeli) Nägeli							+				
Algae	<i>Achnanthes</i> Bory de Saint-Vincent sp.		+		+			+			+
	<i>Amphora</i> Ehrenberg ex Kützing sp.				+						
	<i>Chlorella</i> M.Beijerinck sp.	+	+					+	+		
	<i>Chlorococcum minutum</i> R.C.Starr					+		+	+	+	
	<i>Dynobryon</i> Ehrenberg sp.							+			
	<i>Hantzschia amphioxys</i> (Ehrenberg) Grunow				+			+			
	<i>Oedogonium</i> Link ex Hirn sp.				+						
	<i>Pediastrum duplex</i> Meyen							+			
	<i>Stichococcus</i> Nägeli sp.							+			
<i>Trentepohlia umbrina</i> (Kützing) Bornet	+					+				+	



Fungi	<i>Acrogenospora sphaerocephala</i> (Berk. & Broome) M.B. Ellis									+	
	<i>Alternaria</i> Nees ex Wallroth sp.		+						+		
	<i>Aspergillus niger</i> Tiegh	+									
	<i>Aspergillus ochraceus</i> Micheli	+	+								
	<i>Cladosporium</i> Link ex Gray sp.	+							+	+	+
	<i>Curvularia lunata</i> (Wakker) Boedijn									+	
	<i>Fusarium</i> Link ex Gray sp.	+							+		
	<i>Penicillium</i> Link spp.	+									+
	<i>Rhizopus stolonifer</i> (Ehrenb.) Vuill			+	+		+			+	
	<i>Trichoderma</i> Persoon ex Gray sp.			+		+	+	+	+	+	+
	<i>Ulocladium</i> Preuss sp.								+		
	Total	7	7	3	7	2	5	24	9	3	6

### *Identified photosynthetic organisms*

Algological analysis showed that photosynthetic component of the examined biofilms samples were composed of 16 cyanobacterial and 10 algal taxa in total. The highest diversity of SAB forming cyanobacteria, a total of 12 taxa, was documented on corroded metal surface, while no cyanobacteria were detected on mortar, putty and wooden substrata. *Porphyrosiphon* sp. was the most frequently encountered cyanobacterium, detected as photosynthetic component of 3 different biofilms. On the other hand, the highest diversity of algae was noted on corroded metal, while no algal taxa was recorded on porous sand. Out of 10 identified algae, most frequently encountered were diatom *Achnanthes* sp. and green algae *Chlorella* sp. and *Chlorococcum minutum*, each found on 4 different substrata (Figure 2).

### *Identified fungi*

From all the samples analyzed, 11 fungal taxa were identified. In contrast to photosynthetic microorganisms, fungi were detected in all examined biofilm samples. These fungi belonged to the genera *Acrogenospora*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Rhizopus*, *Trichoderma*, and *Ulocladium* (Figure 2). The most frequently encountered micromycete was *Trichoderma* sp. found on 7 substrata, followed by *Cladosporium* sp. and *Rhizopus stolonifer*, detected as heterotrophic biofilm components on 4 different substrata. Other identified fungi were detected sparingly.

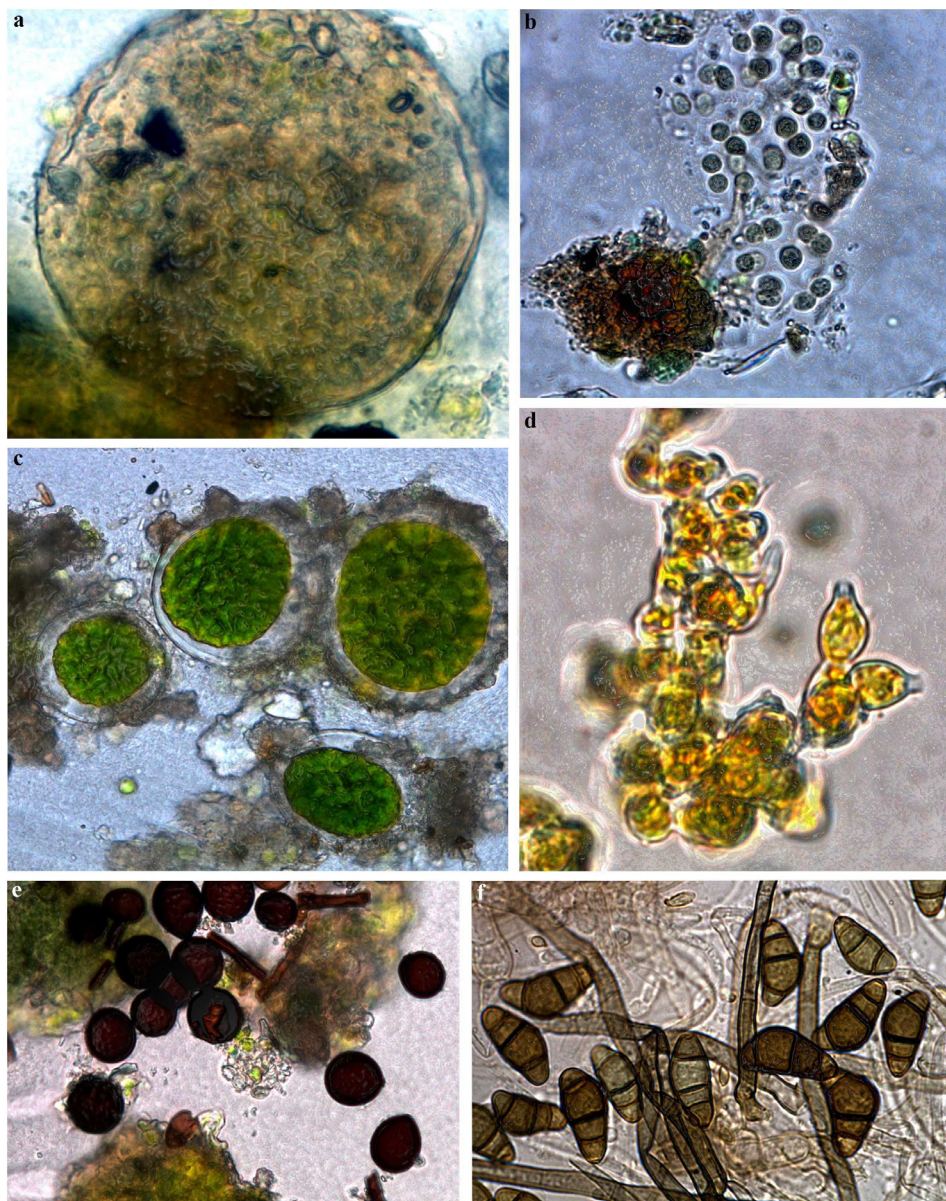


Figure 2. SAB forming microorganisms: a. *Nostoc* sp.-colony; b. *Aphanothece pallida*-colonies (cyanobacteria); c. *Chlorococcum minutum*; d. *Trentepohlia umbrina* (algae); e. *Acrogenospora sphaerocephala*-conidia; f. *Curvularia lunata*-conidia (fungi)

Investigation of microbial communities present in dense layers of highly developed sub-aerial biofilms, documented on all examined substrata within greenhouse, showed high diversity of SAB forming cyanobacteria, algae and

fungi. This result was expected since favourable conditions for development of microorganisms, such as adequate illumination, high humidity levels and constant temperature are present in greenhouse environment. However, in regard to the examined substrata, substantial differences in taxa diversity were detected. The highest diversity of biofilm forming microorganisms, a total of 24 taxa, was documented on the corroded metal surface. Constant moistening of metal substratum, due to frequent watering of plants, is the main cause of a high diversity of phototropic microorganisms and the presence of typically aquatic algae *Dynobryon* sp. and *Pediastrum duplex*. Such conditions facilitate metal corrosion since favorable environment, for colonization by heterotrophic bacteria and fungi, was established. Presence of these aerobic microorganisms results in decrease of oxygen levels beneath biofilm and allows development of anaerobic microbiota. The difference in oxygen concentrations beneath and around microbial biofilm generates an electrochemical potential and electron flow resulting in metal biodeterioration (Gu and Mitchell, 2006). In addition, fungi *Alternaria* sp., *Cladosporium* sp., *Trichoderma* sp., and *Ulocladium* sp. produced dark pigments that bound to the substratum particles and formed aesthetically detrimental discoloration of metal surface.

In regard to taxa diversity, microbial community present on nylon substratum is second to the corroded metal. Highly dense green biofilm was documented on surface of nylon sheets covering windows of greenhouse. Although synthetic in nature, this substratum proved suitable for development of biofilm. This is to a great extent due to high humidity, present in greenhouse interior, and adequate lighting, which comes through windows. However, adverse environmental conditions, such as high temperature and UV radiation, are also present. Nonetheless, SAB forming microorganisms are present in high diversity due to many adaptations for surviving UV exposure and high temperatures. All identified cyanobacterial taxa have gelatinous sheaths, composed of polysaccharides, which act as a water reservoir and play a role in adhesion to the substratum (Macedo *et al.*, 2009; Keshari and Adhikary, 2013). Additionally, scytonemin, UV absorbing yellow-brown pigment, accumulates in the extracellular sheaths of cyanobacteria upon exposure to solar radiation (Balskus and Walsh, 2008). On the other hand, identified dematiaceous fungi produce fungal melanin which protects them from UV light (LJaljević Grbić *et al.*, 2010). These microorganisms may be responsible for biodeterioration of nylon substrata. However, little is known about microbial degradation of synthetic polymers, due to their relatively recent discovery and very slow rate of degradation in natural habitats. Degradation generally depends on chemical structure, molecular weight, crystallinity and physical form of polymer, but environmental conditions may determine the dominant groups of microorganisms that play a role in polymer degradation (Gu and Mitchell, 2006). In this sense, it is important to note the presence of dematiaceous hyphomycete *Curvularia lunata* only on the surface of nylon substratum. Species of this genera were earlier reported to secrete extracellular enzyme-like factor, with esterase properties, which degrade ester-based polyurethane in a polyurethane-agar clearing assay (Crabbe *et al.*, 1994).



Samples of biofilm from clay substratum were characterised by presence of 3 typically aquatic diatoms: *Achnanthes* sp., *Amphora* sp., and *Hantzschia amphioxys*. In general, diatoms are among the most successful contemporary groups of photosynthetic microorganisms that occur in virtually every environment containing water (Vanormelingen *et al.*, 2008). This holds true not only for freshwater and marine habitats, but also for temporary aquatic and moist soil habitats. Constant moistening of clay and other substrata, due to frequent watering of plants in greenhouse, created favorable conditions for colonization by diatoms.

Highly developed biofilms were documented on surfaces of all examined substrata. However, in regard to documented taxa, mortar and putty are considered much less diverse, with only 2 and 3 identified taxa, respectively. Nonetheless, algological and mycological analyses of all biofilm samples showed a large number of cyanobacteria (*Aphanothece pallida*, *Gloeocapsa* spp., *Nostoc* sp.), algae (*Chlorococcum minutum*, *Trentepohlia umbrina*), and fungi (*Alternaria* sp., *Cladosporium* sp., *Curvularia lunata*, *Trichoderma* sp. and *Ulocladium* sp.), causing discoloration and biodeterioration of the substrata (Ljaljević Grbić *et al.*, 2009, 2010). This finding is consistent with documented symptoms on the surfaces of examined substrata.

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## МИКРООРГАНИЗМИ У САСТАВУ БИОФИЛМА С РАЗЛИЧИТИХ СУПСТРАТА СТАКЛЕНИКА БОТАНИЧКЕ БАШТЕ „ЈЕВРЕМОВАЦ”

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**РЕЗИМЕ:** Испитиван је диверзитет цијанобактерија, алги и гљива у биофилму с 10 различитих супстрата из стакленика Ботаничке баште „Јевремовац“. Од 37 документованих таксона, идентификовано је 16 цијанобактерија и 10 алги. Преосталих 11 таксона припадају „Петом царству“. Највећа разноврсност микроорганизама, укупно 24 таксона, забележена је у биофилму на кородираној металној површини, док је значајно нижи број таксона регистрован на осталим испитиваним супстратима. Цијанобактерија *Porphyrosiphon* sp., дијатома *Achnanthes* sp. и зелене алге *Chlorella* sp. и *Chlorococcum minutum* су најчешће фотосинтетичке компоненте биофилма. У свим испитиваним узорцима *Trichoderma* sp., заједно са *Cladosporium* sp. и *Rhizopus stolonifer* су најчешће идентификоване гљиве.

**КЉУЧНЕ РЕЧИ:** алге, биофилм, гљиве, стакленик, цијанобактерије