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SPECIAL ISSUE

Microbial diversity on a marble monument: a case study

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Abstract In the presented case study, ascomycete fungi and green algae on a marble monument were identified by comparisons of the 18S rRNA gene sequences, which were obtained from DNA either from environmental samples or from enrichment cultures. The organisms were found to be responsible for either black or green surface coverings on different areas of the monument surface. Most fungi were related to plant-inhabiting genera, corresponding to a heavy soiling of the marble surface with honeydew. Whereas green algae of the genera *Stichococcus*, *Chloroidium* and *Apatococcus* were found to be dominant in all samples, isolates of two additional genera were recovered only from enrichment cultures. A reference strain of *Apatococcus lobatus* and an isolate of *Prasiolopsis* sp. were investigated with respect to putative surface adhesive structures of the cell envelope. The *Prasiolopsis* cell walls were covered with a thin adhesive exopolysaccharide layer involved in biofilm formation.

Keywords Marble monument · Biofilm · Ascomycete fungi · Green algae · Cell wall · Exopolysaccharide

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Introduction

Biodeterioration of dimension stone primarily affects material surfaces. Some endolithic organisms actively penetrate the surface and are also found in layers up to several millimeters underneath the surface (e.g., Ascaso et al. 1998; Hoppert et al. 2004a). Though several important pro- and eukaryotic organisms have already been recognized as deteriorative agents, up to now little is known about the whole microbial community (see Gorbushina 2007 and Macedo et al. 2009 for review). It must be expected that a microbial biofilm is composed of at least several dozens of species. These various organisms act in different ways on the material; a whole spectrum of deteriorative activities may be expected: from neutral (or even protective, e.g. Zuo et al. 2005) to deteriorative by penetration of the surface (Warscheid and Braams 2000; Kemmling et al. 2004). It remains difficult to assign the deteriorative activity to a certain microbial species within a biofilm. Microbial activity also varies over seasons and is influenced by rainfall, surface cleaning or input of nitrogen, phosphate and other essential compounds.

The present case study aims at the identification of organisms by two complementary approaches: analyses of 18S rDNA sequences (either directly from environmental DNA or via enrichment culture) and isolation of strains. The former approach is inevitable for elucidation of microbial diversity, since most (pro- and eukaryotic) microorganisms

are unculturable. The latter, "classical" approach underestimates the diversity as it recovers too few of the actually present species, but has two advantages. It enables studying the physiology of organisms in pure culture and allows recovering and identification of species that are present on the surface in such low numbers of individuals that they escape DNA extraction and sequencing. These species may actually not have an important role in the biofilm at the time of sampling, but may be present as resting stages and become more abundant when the environmental conditions (e.g., insolation, humidity) change.

For the present case study, a marble sculpture, which is part of the monument "Gegendenkmal" (Fig. 1a; Hamburger Dammtordamm, created by Alfred Hrdlicka 1983–1986), was selected. The object is covered with grayish and black crusts of hitherto unknown origin as well as greenish (obviously algal) biofilms. The aim of this case study was the application of both molecular and

Fig. 1 The monument and macroscopically visible surface stains. **a** The monument "Gegendenkmal", created by Alfred Hrdlicka 1983–1986, located in Hamburg-Altona. The marble sculpture at the left is nearly completely covered with a *dark gray/black* stain. **b**, **c** Part of the sculpture "Hamburger Feuersturm" with a *green* (**b**) and *dark-grayish* (**c**) surface stain, as marked by *arrows*. (**d**, **e**) Thin petrographic sections perpendicular to the surface from an area as depicted in (**c**). Small fragments, embedded in a *dark* matrix, adhere to the surface (*arrows*, **d**). Microfractures along grain boundaries, filled with a *dark* matrix (*arrows*)

enrichment/isolation approaches to identify the organisms and to characterize cell wall features that are possibly relevant to biofilm formation. Here, we focus on green algal and fungal organisms. Members of these groups dominate the biofilm, from the first microscopic inspection of the surface, with by far highest biomasses.

Materials and methods

Sampling

The sampling site was in Hamburg Dammtor (53°33′43″N, 9°59′27″E). Samples (approximately 100 µl dry volume) were collected in May 2008 from an SSW exposed part of the sculpture called "Hamburger Feuersturm". The samples were taken from two sites as depicted in Fig. 1b, c. Areas of 1 cm^2 from a green surface covering (Fig. 1b, sample A) and from a dark gray/black covering (Fig. 1c, sample B), respectively, were scraped off with a sterile scalpel. The samples were stored at ambient temperature in sterile 2-ml reaction tubes.

Cultivation and isolation

For the cultivation of microalgae, small amounts of the biofilm samples were either directly plated on agarized (1.5%) culture medium MIEB12 (Schlösser 1994) in Petri dishes, or inoculated in 10 ml volumes of liquid MIEB12 medium in culture tubes (enrichment culture). Cultures were kept under continuous illumination (25 µmol photons m^{-2} s^{-1}, white fluorescent tubes) and 18°C for 4 weeks. From the liquid cultures, 100 µl aliquots were then plated on solid MIEB12 medium and incubated under the same conditions as for liquid media. Single colonies of different appearance were selected and transferred on fresh agar plates until unialgal cultures were obtained.

A reference stain, *Apatococcus lobatus* SAG 2037, was taken from the Culture Collection of Algae (SAG; Georg-August-Universität Göttingen, Germany) and cultured on agarized or liquid Bold's Basal medium with vitamins (Schlösser 1994).

Nucleic acid extraction, PCR, cloning and sequencing

DNA was extracted from the environmental samples as well as from the liquid cultures. All steps were performed with sterilized (autoclaved, nuclease-free) reagents. For DNA extraction, equivalents of 20–50 µl packed cell volume, either obtained from the original biofilm or from liquid cultures, were used. The biofilm samples or pelleted cells were resuspended in 100 µl lysis buffer (Invisorb Spin Plant Mini Kit, Invitek, Berlin, Germany). After one volume of resuspended cells was mixed with an approximately equivalent volume of glass beads (425–600 µm diameter; acid-washed beads, Sigma–Aldrich, St. Louis, MO, USA) and vortexed briefly, the cells were mechanically disrupted by shaking in a Minibeadbeater (Biospec, Bartlesville, OK, USA) in several intervals of 30 s and one interval of 50 s at 5,000 rpm. DNA was then extracted with the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) in the extraction buffers, following the manufacturer's instructions. Results were checked on a 1% (w/v) agarose gel. Isolated DNA was stored at −20°C until further processing. The eukaryote-specific primer combinations NS1+18L and NS1+LR 1850 were used (Table 1) to amplify the 18S and the 18S with adjacent ITS-1/5.8S/ITS-2 regions of rDNA, respectively.

About 30 ng of the extracted DNA was used as template. The amplification reaction mixture (50 µl) contained each deoxynucleotide triphosphate at a concentration of 0.1 mM, 5 µl of tenfold concentrated reaction buffer, 2 mM $MgCl_2$, 0.2 µM primers, 2 U of Taq DNA polymerase (reagents and manufacturer's protocol: Bioline, Luckenwalde, Germany) and 4% (v/v) dimethyl sulfoxide (DMSO)-solution. Polymerase chain reaction was carried out on a PTC 200 thermocycler (MJ Research, Waltham, MA, USA) using the following program for the primer pair NS1+LR1850: initial denaturation at 95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 40 s, annealing at 52°C for 90 s, extension at 72°C for 90 s, followed by 6 cycles of denaturation at 94°C and final extension at 72°C for 2 min. For the primer pair NS1+18L, denaturation was for 1 min, annealing for 45 s, extension for 3 min, the additional 6 cycles were omitted and final

Table 1 Primers used in this study

Primer	Sequence 5′-3′	Reference
NS1 (forward)	GTAGTCATATGCTTGTCT	White et al. (1990)
18L (reverse)	CACCTACGGAAACCTTGTTACGACTT	Hamby et al. (1988)
LR1850 (reverse)	CCTCACGGTACTTGTTC	Friedl (1996)
M13F (forward)	TGTAAAACGACGGCCAGT	Invitrogen
M13R (reverse)	GGCAGGAAACAGCTATGACC	Invitrogen
895R (sequencing primer)	GAGGTGAAATTCTTGGATTT	SAG

extension was for 7 min. The PCR products were purified using the Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany). Aliquots (2 µl) of purified amplicons were analyzed by electrophoresis on a 1% (w/v) agarose gel. Cloning of the PCR product was performed with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the pCR2.1-TOPO vector. Ligations were transformed into competent cells of *Escherichia coli* TOP 10, as supplied by the manufacturer. In the plasmid screening, white *E. coli* colonies containing correct DNA insertions were identified by direct amplification of the inserted DNA fragment with a vector-specific primer set M13F/M13R (Table 1).

Clones were cultivated overnight in LidBac reaction tubes (Qiagen, Hilden, Germany) with 1 ml LB medium containing 100 µg ampicillin. Plasmid DNA was prepared from the clones with a NucleoSpin-Plasmid kit (Macherey and Nagel, Düren, Germany) following the manufacturer's instructions.

Sequencing reactions were performed with a Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 3100 (Applied Biosystems) automated sequencer. All clones were sequenced with the 18S sequencing primer 895R (Table 1) resulting in about 600 bp sequences. Sequences were processed using the sequence analysis program SeqAssem (Sequentix, Klein Raden, Germany) and manually aligned using the program BioEdit v7.0.5.3 (Meusnier et al. 2008). The final sequences were compared by BLASTn analysis [National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov].

Light and electron microscopy

For fluorescence light microscopy, cells taken from unialgal or pure cultures were marked with concanavalin A, coupled to fluorescein isothiocyanate (Con A-FITC; Sigma–Aldrich). The dye was applied in 50 mM potassium phosphate buffer, supplemented with 10 µM magnesium chloride and 10 µM calcium chloride at a dilution of 1/1,000 of the original stock solution. The sample was inspected under a fluorescence light microscope (Axioscope, Zeiss, Göttingen, Germany; excitation wavelength 495 nm, emission wavelength 517 nm, Zeiss filter set 09).

Petrographic thin sections of approximately 30 µm in thickness were performed according to established procedures (Adams et al. 1984) and visualized by standard bright field microscopy.

For electron microscopy, cells from unialgal or pure cultures were harvested by centrifugation at 10,000×*g*, resuspended in 50 mM potassium phosphate buffer, chemically fixed in 0.5% (w/v) formaldehyde and 0.3% (w/v) glutaraldehyde solution for 90 min at 0°C, dehydrated in a graded methanol series and embedded in Lowicryl K4M resin (Roth et al. 1981; Hoppert and Holzenburg 1998). Resin sections of 80–100 nm thickness were cut with glass knives. Localization studies were performed with the lectin concanavalin A (Sigma–Aldrich), coupled to colloidal gold (Con A-Gold), as already described (Kämper et al. 2004). In brief, sections were incubated on drops of dilutions (1/10, 1/100, 1/1,000) of the Con A-gold marker for 90 min, then washed for 5 min per step for three times on drops of PBS containing 0.01% (v/v) Tween 20. Finally, the sections were stained with 0.5% (w/v) phosphotungstic acid, pH 7.0, for 3 min. Electron microscopy was performed in a Zeiss EM 902 transmission electron microscope (Zeiss SMT, Oberkochen, Germany), equipped with a 1 K digital camera, at 80 kV acceleration voltage and at calibrated magnifications.

Results

Diversity of fungal and algal organisms

The monument "Gegendenkmal" consists of several bronze and marble sculptures. In this study, the sculpture "Hamburger Feuersturm", made of Carrara marble, was under investigation. The sculpture showed a mixture of greenish and grayish/black stains (Fig. 1), as well as green layers on the fracture surfaces of chips and scales (cf. Warscheid and Braams 2000). The sculpture was manufactured from Bianco Carrara C, a pure calcite marble, as determined by X-ray diffractometry (data not shown).

Thin petrographic sections, perpendicular to the surface, show open grain boundaries between the calcite crystals in the marble microstructure (Fig. 1d, e), which illustrates the increased porosity and a slight sugar-like crumbling of the marble surface. The grayish stain, mainly on the top parts of the marble sculptures, was the most obvious feature (Fig. 1a, c). In petrographic sections, the stain appears to be homogeneous, slightly red-brown and translucent. It encloses crystals on the marble surface (Fig. 1d) and infiltrates the surface along open grain boundaries (Fig. 1e). Though the cause of this heavy soiling could not be identified, it was observed that honeydew covered the sculptures in the affected areas. The honeydew was dripping off a plane tree canopy (*Platanus* × *hispanica*), placed directly above the monument, as well as a lime tree canopy (*Tilia platyphyllos*) nearby. Another obvious feature was represented by green stains of algal biofilms, primarily growing in cavities, where water cannot drain off fast. To investigate the participation of microorganisms in these surface coverings, material from approximately 1 cm^2 surface was used for further processing. Green layers under scales were not enclosed in this study, because they

represented just a small fraction of the (putatively) biogenic stains. Sample A was taken from a green algal biofilm (Fig. 1b) and sample B from the black covering as depicted in Fig. 1c.

The samples were first inspected by light microscopy to verify the presence of dominant species. Actually, green algal (sample A) and fungal (sample B) morphotypes dominated the microbial biomass of the biofilm (Fig. 2a, b). Thus, our further studies aimed at the identification of the species from both these groups. Most of the algal morphotypes correspond to *Apatococcus* and *Chloroidium ellipsoideum* (Darienko et al. 2010). No stratification of the biofilms could be observed: filamentous fungi (if present) and green algae were interwoven in a homogeneous surface covering. In enrichment cultures, inoculated with samples from the original biofilm, besides *Chloroidium*-morphotypes, rod-shaped *Stichococcus*-like cells dominated (Fig. 2c). Unialgal cultures could be obtained for some of the dominant *Stichococcus* and *Apatococcus* like morphotypes (cf. Fig. 3a, b). In addition, a xanthophyte alga (Fig. 3c) and a *Prasiolopsis* (*Pseudopleurococcus*)-like morphotype (Fig. 3d; see below) could be isolated.

Sequences obtained from clone libraries of environmental DNA samples also recovered *Stichococcus* (*S. mirabilis* related) and *Apatococcus*, however, failed to detect *Prasiolopsis* and xanthophytes (Table 2). Instead, *Trebouxia* (a frequent lichen alga) was found. Besides the dominating *Stichococcus* spp. algae, a variety of ascomycete fungi was found exclusively in sample B. Among the fungi, members of the ascomycete genera, *Batcheloromyces*, *Teratosphaeria*, *Thelocarpon* and *Sarcinomyces*, were recovered, each with more than one clone. *Batcheloromyces*, *Teratosphaeria* and *Guignardia* are known as plant-inhabiting ascomycetes (Crous et al. 2004). *Thelocarpon* represents a lichenicolous ascomycete genus. The genus *Sarcinomyces* describes black yeasts from various habitats (Cooke 1961).

The clone libraries from the enrichment cultures showed lower diversities. *Stichoccoccus bacillaris*-like algae were dominant (Table 3) and no fungal organisms could be detected. It has to be stated, nevertheless, that not all organisms could be assessed by the clone bank analysis. The observed xanthophycean algae as well as *Prasiolopsis*, which were observed in the cultures, were not recovered by clone libraries from environmental samples. The identity of the *Prasiolopsis* genus was confirmed by 18S rDNA sequencing of preparations obtained from unialgal cultures.

Cell surface features of selected species

The branched filamentous *Prasiolopsis* was selected for further morphological studies. The organism was compared with a contrasting morphotype, represented by *Apatococcus lobatus* SAG 2037, which forms irregular packages consisting of several dozens of cells (Figs. 3b, 4a). These algae represent the most different biofilm morphotypes in this study, i.e., unicellular coccoid and

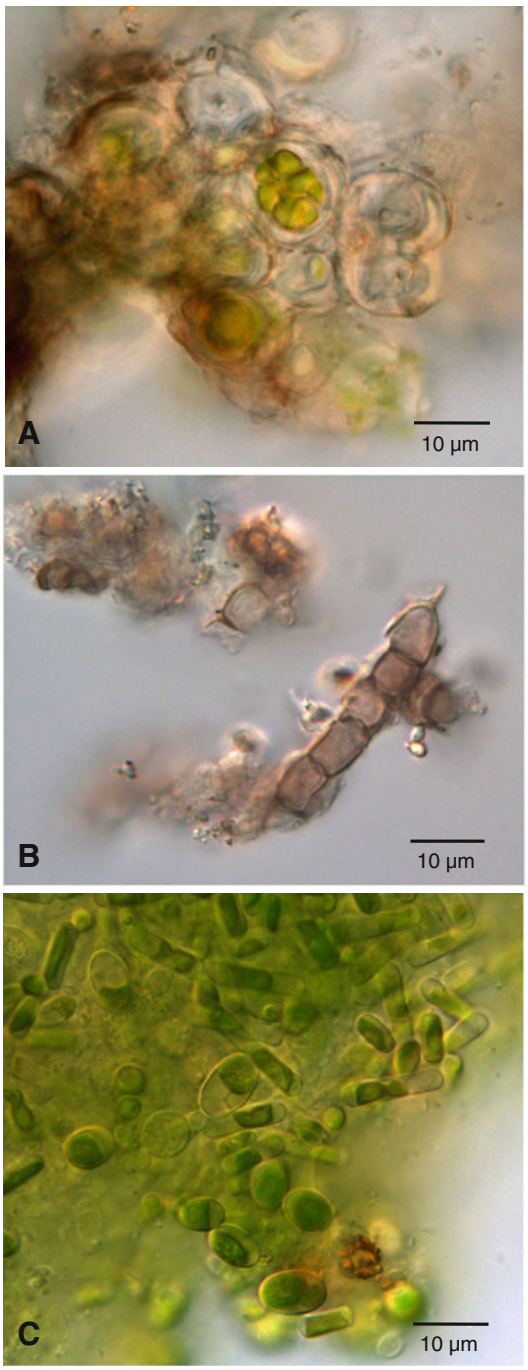

Fig. 2 Microscopic analysis of environmental samples and enrichment cultures. **a** Agglomeration of coccoid algae taken from the marble surface. **b** Ascomycete fungal morphotype (putative conidial fragment) taken from the surface. **c** Unicellular green algal morphotypes in an enrichment culture

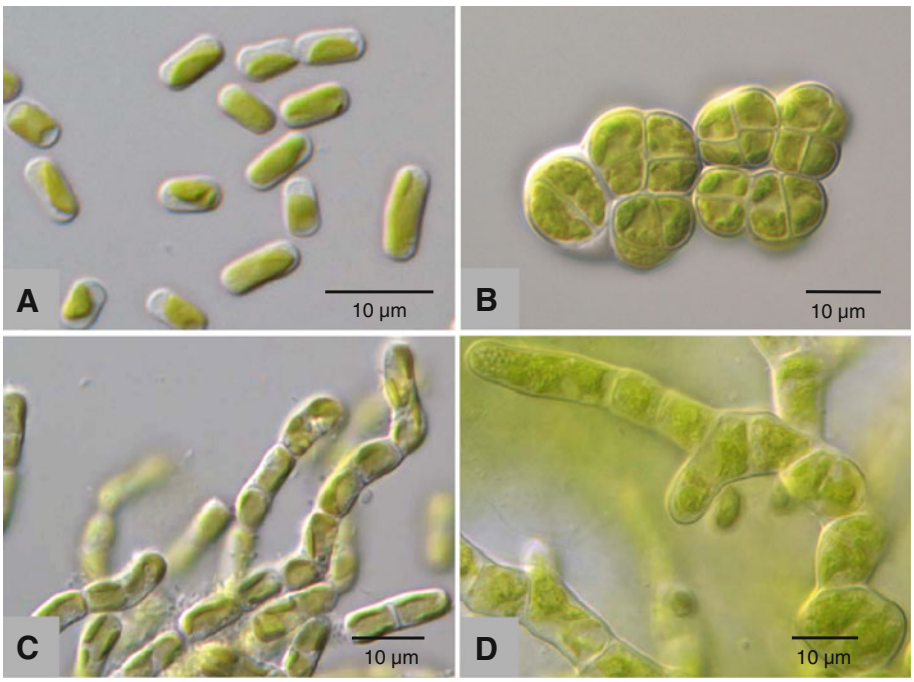

Fig. 3 Morphotypes of investigated algal stains. **a** *Stichococcus* sp., **b** *Apatococcus lobatus* SAG 2037, **c** a xanthophycean algae, **d** *Prasiolopsis* sp.

branched filamentous (Figs. 3b, d, 4). With respect to biofilm formation, we were particularly interested in cell surface features that are involved in adhesion, such as secreted polysaccharides (e.g., Tsuneda et al. 2003). In search of such features, polysaccharide-specific stains were applied (Figs. 4, 5). In ultrathin sections of the algal cell walls, a thin irregular layer on the surface of the *Prasiolopsis* cell walls was observed (Fig. 4e, arrows), whereas the cell walls of *Apatococcus* did not show any additional layers (Fig. 4b). To detect possible heteropolysaccharides of the cell envelope, staining with labeled concanavalin A (Con A) was applied. In both algae, the cell periphery showed certain fluorescence signals (Fig. 4a, d). Localization with Con A-gold revealed, at electron microscopic resolution, a scattered distribution of gold markers over the whole cell wall section of *Apatococcus* (Fig. 4c), and a very distinct marked outer layer in *Prasiolopsis* (Fig. 4f). This accounts for a thin layer of exopolysaccharides (EP) in *Prasiolopsis.* This layer appears to be involved in adhesion of separate *Prasiolopsis* filaments as depicted in Fig. 5. Here, the cells adhere to each other by the thin EP layer (Fig. 5b).

Discussion

Our case study aims at the elucidation of surface stains caused by microbial impact. Besides general climatic features (such as precipitation, insolation or temperature), especially local factors (the effect of honeydew) determine the biofilm formation and its taxonomic composition.

The sampling site in Hamburg experiences an oceanic climate, with average annual precipitation of 774 mm and an average annual temperature of 9°C. The average temperature in May 2008, however, was approximately 15°C (3°C higher than the long-time average for May). Precipitation was just about 25% of the long-time average for May (54 mm) (data were taken from Deutscher Wetterdienst, Offenbach). Thus, the biofilms developed under relatively warm and dry conditions. It is, however, still difficult to draw a relation between climate and the detected organisms: most of the detected species are distributed worldwide, with seemingly broad ecological amplitude, a finding typical for most microorganisms known so far (c.f. Finlay and Esteban, 2004). Thus, a "biogeography" of microbial species is yet to be revealed (Hedlund and Staley 2004; Martiny et al. 2006). From the data presented here, however, one feature is obvious. Molecular analysis of 18S rDNA clone libraries from sample B revealed a high diversity of ascomycete fungi, as compared with sample A, dominated by algae (Table 2). Sample B was taken from an area intensively soiled by honeydew. Sample A was taken from a cavity (Fig. 1b), where, due to the complex geometry of the sculpture, immediate soiling with honeydew can be excluded. Here, the photoautotrophic algae are dominant. These cavities will retain rainwater for longer time than the exposed surfaces. Rainwater may also transport some dissolved carbohydrates (see below) from the honeydew layer to the cavity but, in toto, the conditions are obviously more favorable for algae than for ascomycetes.

Intriguingly, among the most frequently found clones from sample B, the closest related species are known as

plant associated or plant pathogenic. *Batcheloromyces proteae* and *Teratospora* (*Mycosphaerella*) *microspora* are (opportunistic) pathogens, which are causative agents of black leaf spot disease (Crous et al. 2004). Also *Spencermartinsia* sp. is a plant-inhabiting fungus and an opportunistic phytopathogen (Phillips et al. 2008), as well as *Xenomeris raetica*. Members of the genus *Xenomeris* are possibly infectious agents of tree canker (Jasalavich et al. 2000). *Phialophora* comprises plant- and human-pathogenic species, as well as saprophytic, wood-decaying non-pathogens (Abliz et al. 2004). *Guignardia magniferae* is an endophytic, but not necessarily a plant-pathogenic, fungus (Suryanarayanan et al. 2004). Undoubtedly, the plant-associated fungi were transferred from the tree canopies to the marble surface, by honeydew droplets. Honeydew is a carbohydrate-rich secretion mainly consisting of monosaccharides, as well as the trisaccharide melezitose (*O*-α-D-glucopyranosyl-(→3)-*O*-*β*-D-fructofuranosyl-(2→)-α-D-glucopyranoside), which can be easily used by fungi and other microorganisms as growth substrates (Fischer et al. 2002). The concentration of amino acids in honeydew is relatively low (in the range of 3–20 nmol/µl), which is a growth-limiting factor. However, the overall nitrogen input (also from atmospheric sources) is sufficient for the development of plant-associated fungi: such as other oligotrophic fungi, they are well adapted to the plant biomass with wide C/N ratios (Wainright et al. 1993 and references therein).

Massive colonization of black-pigmented ascomycete fungi ("black fungi", Dematiaceae) has been frequently observed in conjunction with honeydew coverings of surfaces (e.g., Crozier 1981; Gerson 1975). On leaves of affected plants, colonization is referred to as sooty mold. Sooty mold is also a common disease on plane and linden trees (Hughes 1976). Among other genera, also *Aureobasidium* species have been described for the normal phyllosphere as well as for sooty mold. *Aureobasidium* is not just a plant-associated ascomycete. Members of this genus, as well as the detected *Sarcinomyces*, frequently occur on rock and dimension stone surfaces (Simonovicova et al. 2004; Wollenzien et al. 1997). Organisms of this group have been identified as deteriorative rock-dwelling agents on natural and dimension stone (Gorbushina et al. 1993; Gorbushina and Krumbein 2000). The black pigmentation is attributed to the ultraviolet-protective melanin (Bell and Wheeler 1986). In fungi, melanin is synthesized via different pathways, frequently from acetate via 1, 8-dihydroxynaphthalene as intermediate. The resulting phenolic polymer is deposited in- or outside the fungal cell wall

Table 2 18S rDNA analysis of clones obtained from environmental samples

Closest relative species	Number of clones		% sequence similarity to closest relative species
	(sample A)	(sample B)	
Algae			
Stichococcus mirabilis	10	24	98–99
Uncultured *Trebouxia* sp.	1	1	97–98
Apatococcus sp.	2	–	98–99
Stichococcus sp.	1	1	99
Stichococcus jenerensis	1	–	98
Chloroidium mirabilis	1	–	98
Stichococcus bacillaris	1	–	96
Acomycete fungi			
Batcheloromyces proteae		5	98–99
Teratosphaeria microspora		3	96
Thelocarpon laureri		3	95–97
Sarcinomyces sp.		2	93–97
Guignardia mangiferae		1	100
Phaeoramularia hachijoensis		1	98
Phialophora sp.		1	98
Conisporium perforans		1	97
Spencermartinsia sp.		1	97
Xenomeris raetica		1	97
Symbiotaphrina kochii		1	97
Mycocalicium polyporaeum		1	96
Aureobasidium pullulans		1	95
Harpidium rutilans		1	95
Pseudofusicoccum stromaticum		1	93

Table 3 18S rDNA analysis of clones obtained from enrichment cultures

Closest relative species	Number of clones		% sequence similarity to closest relative species
	(sample A)	(sample B)	
Algae			
Stichococcus bacillaris	21	12	98–100
Chloroidium ellipsoideum	4	2	98–100
Chloroidium angustoellipsoideum	3	1	99
Stichococcus mirabilis	–	3	98
Stichococcus deasonii	2	–	100

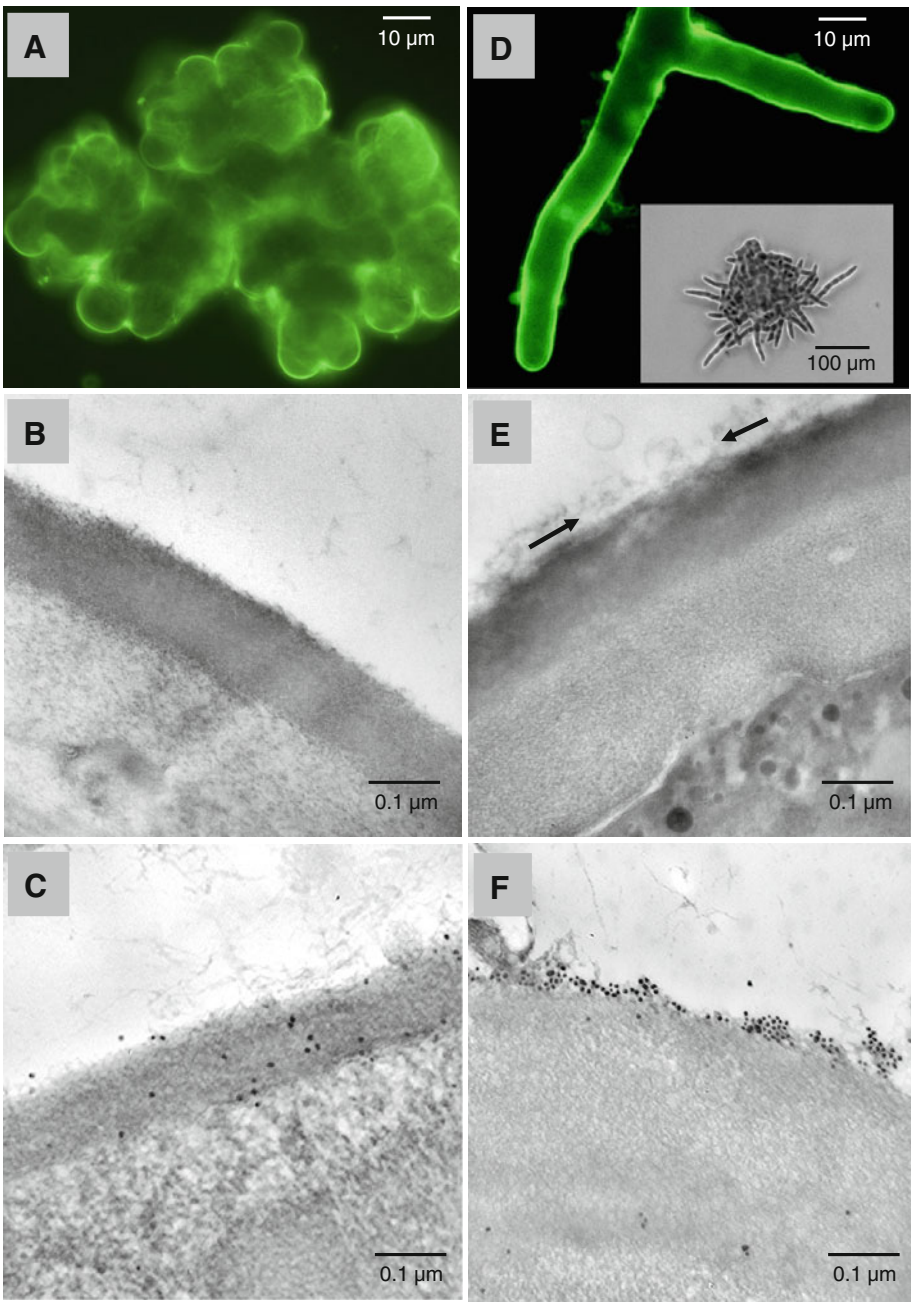

Fig. 4 Light and electron microscopy of lectin-labeled cells. **a** Fluorescence light microscopy of a Con A-FITC-labeled *Apatococcus* aggregate. **b** Ultrathin section of an *Apatococcus* cell wall. **c** Con A-gold labeled ultrathin section of an *Apatococcus* cell wall. *Dark dots* represent the colloidal gold marker. **d** Fluorescence light microscopy of Con A-FITC-labeled *Prasiolopsis* filaments. The inset shows a typical aggregate (phase contrast image). **e** Ultrathin section of a *Prasiolopsis* cell wall. The thin exopolysaccharide layer is marked by *arrows*. **f** Con A gold-labeled ultrathin section of a *Prasiolopsis* cell wall

(Bartnicki-Garcia 1968). Hence, the dark stain is difficult to handle. Though superficial soiling, even black gypsum crusts on top of a surface are removable with mild chemical or biological agents (e.g., Polo et al. 2010), the organisms and stains may also penetrate the genuine marble surface (cf. Fig. 1e). When these cell wall remnants cannot be removed mechanically, or by application of mild detergents, rather harsh chemical treatment, e.g., with hydrogen peroxide as strong oxidant, is necessary to destroy the melanin (Korytowski and Sarna 1990).

According to the clone library data, the group of plant-associated ascomycetes dominates over the rock-inhabiting genera. Though even sequencing clone libraries to saturation (which was not done here) does not reflect the real species diversity exactly (Jeon et al. 2008), we find in our case study a clear tendency for the presence of "allochthonous", not genuinely rock-inhabiting fungi. The organisms grow in the honeydew cover on the marble sculpture, whereas the "autochthonous" organisms do not benefit from the honeydew in the same way.

In contrast, the algal species of the genus *Stichococcus* are typical colonizers of stone surfaces (cf. e.g., Michailyuk 2008). It is noticeable that, though the algae are photoautotrophic, they may also benefit from external organic

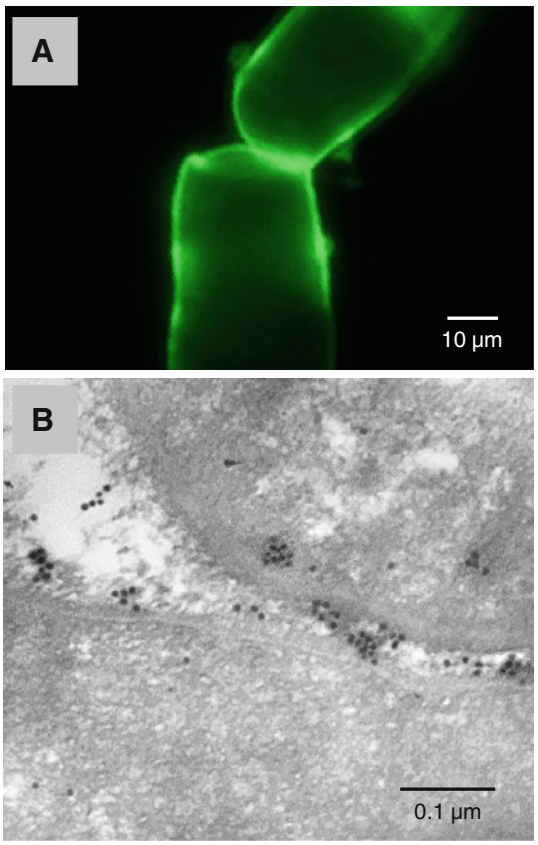

Fig. 5 Agglutination of *Prasiolopsis* filaments. **a** Fluorescence light microscopy of two attached filaments. **b** The same situation as depicted in **a** visualized by electron microscopy. Two attached filaments agglutinate via a thin exopolysaccharide layer, labeled with the Con A-gold maker

nutrients, especially from sucrose (e.g., Samejima and Myers 1958). Growth enhancement by carbohydrates has also been shown for *Stichococcus mirabilis* (Mattox and Bold 1962). This may be one reason for the abundance of this algal species in a biofilm dominated by diverse fungi. Generally, the most abundant algal species were small unicellular morphotypes, known as pioneer organisms (e.g., Garty 1992; Bellinzoni et al. 2003). Besides these organisms, others must be present in very low numbers of individuals, as a "seed bank" inside the biofilm (Table 3). Hence, their sequences were not present in the clone libraries, derived from environmental DNA. They will become important when environmental conditions change (which is, in fact, the case during enrichment of organisms from biofilm samples in a liquid culture). Some species may even not be detected in clone libraries of enrichment cultures, such as, in our example, *Prasiolopsis* sp., a green alga that could be obtained in unialgal culture. *Prasiolopsis* is a peculiar subaerial alga living on rock or tree bark with multiseriate filamentous or pseudo-parenchymatous thalli (Karsten et al. 2005). Branched filaments are well adapted to those environments where tiny paths and small cavities, as well as impassable (crystalline) particles form a more or less solid substratum (e.g., Ritz and Young 2004). Moreover, the algal exopolysaccharides (EP) facilitate the attachment to a surface as well as agglutination of organisms to each other. Up to now, mainly thick algal EP layers in aquatic biofilms have been described (see Sutherland 2001 for review), where green algae and cyanobacteria are covered with a thick polysaccharide layer. It is also known that EP stabilizes terrestrial (soil) biofilms such as microbiotic soil crusts (Hoppert et al. 2004b; Bowker et al. 2008). The role of EP on dry, solid surfaces is much less known, though it is obvious that the organisms also produce these extracellular polymers. For detection of specific oligosaccharides in EP, marker techniques based on the binding of lectins to certain oligosaccharides stretches are used. The lectin concanavalin A binds to oligomannose-type *N*-glycans. These motifs are common in cell wall polysaccharides and especially in EP of a variety of organisms, including cyanobacteria and eukaryotic algae (Mehta and Vaidya 1978; Tien et al. 2005).

The *Apatococcus* strain does not show a layer adjacent to the cell wall, though the rigid wall itself exhibits a dispersed labeling (Fig. 4c). Heteropolysaccharides of different proportions are common in algal cell walls (Takeda and Hirokawa 1984; Okuda 2002) and can also be expected for *Apatococcus*. *Prasiolopsis*, in contrast, forms a thin layer of just several tens of nm in thickness, distinctively labeled with the concanavalin A-lectin (Fig. 4e, f). Compared with other EP layers from eukaryotic algae (e.g., Leppard 1995), *Prasiolopsis* exhibits even in the hydrated state only a thin EPS coating. This layer may not have more than a gluing function. Up to now, there are no data concerning the interaction between the filaments and a material surface, but it is obvious that single filaments agglutinate via the thin EP layer (Fig. 5). This may be an important feature for the formation of an interwoven meshwork of branched filaments.

Conclusion

In our case study, we examined the microbial diversity on a monument contaminated by honeydew. Remarkably, typical rock-inhabiting fungi appear to be less relevant than plant-inhabiting fungal microorganisms. Less contaminated areas of the sculpture exhibited typical green algal biofilms. Among the algae, unicellular *Stichococcus*-, *Chloroidium*- and *Apatococcus*-related strains were dominant in environmental samples and in enrichment cultures. Other algae, like *Prasiolopsis*, could also be isolated.

These results show the high relevance of an external carbon source (honeydew) for surface colonization by ascomycete fungi and, hence, formation of a dark-stained

biofilm layer. The dark surface stain can only be avoided by removal of the carbon source, e.g., the honeydew, which may be achieved by suitable pest control. Also, an appropriate shaping of the canopy may lead, in this case, to an effect: parts of the sculptures are presently positioned in a way that they are placed rather under the periphery of the tree canopies. Thinning of the crown may not completely prevent, but reduce, the impact of down-dripping honeydew.

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