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STUDY OF EXOPOLYSACCHARIDE-PRODUCING CYANOBACTERIA IN BIOFILMS GROWING ON LITHIC SUBSTRATES AND IN EXTREME ENVIRONMENTS

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1. Introduction

1.1 Biofilms

Biofilms are communities of micro-organisms growing on a substrate, embedded in a slimy matrix. They can be found in natural, technical and medical environments.

Though in the history of microbiology microorganisms have mostly been characterized in their planktonic, freely suspended forms, in recent years the studies of surface-associated organisms, first described by Leeuwenhoek, have grown in number following the repercussions that their growth can have in many anthropogenic fields.

Biofilms are virtually able to grow in a wide range of environments. Most commonly they can be found where there's a combination of moisture, nutrients and a colonizable substrate. They can be defined as an assemblage of microbial cells irreversibly associated (by no means removable with gentle rinsing) and enclosed in a matrix of primarily polysaccharidic material (1).

The architecture of these consortia varies through time and space so that every community can be considered as unique.

Table 1 *Biological and non-biological fractions constituting a biofilm (2)*

Costituents of biofilms

Fraction	***Fraction content***
Extracellular polymeric substances (EPS)	*Polysaccharides, Cationic groups of amino sugars and proteins; anionic groups in uronic acids, proteins, nucleic acids, apolar groups from proteins*
Microbial cells	*Outer membranes (lipopolysaccharides in gram- cells), cell walls*
Minerals	*Precipitates (sulfides, carbonates, phosphates, hydroxides), free and bound metals*
Biogenic particulate material	*Degradation products*
Environmentally relevant substances	*Organic pollutants (e.g. biocides, detergents, xenobiotics), inorganic pollutants (heavy metals)*

Nonetheless, as a common feature, it has to be stated that biofilms cannot be considered as continuous monolayers but as microcolonies of bacterial cells embedded in a saccharidic matrix, which are separated from one another by voids (water channels) which provide for water diffusion of nutrients and gases (1,2).

The major component (97%) of a biofilm matrix is represented by water (3), the remaining being constituted by cells, minerals and extracellular polymers (table 1).

Microbial aggregates grow almost everywhere on earth, being practically omnipresent. They can be found in a range of temperature from -5°C to 120°C, in a range of pH from 0 to 13 and pressures up to 100 MPa dwelling in those habitats that, being inaccessible to many organisms for their constraints, can be defined as 'extreme'.

The formation of a biofilm can be summarily divided in three stages (2). The first is characterized by a weak adhesion of a single cell, thanks to Van der Waals forces, that afterwards becomes stable and irreversible. The attachment is enhanced by substrates with hydrophobic properties and thus also depends on the properties of the molecules on the cell surface: non polar structures tend to bind to hydrophobic sites while extracellular molecules tend to bind to the hydrophilic ones (1). Teflon and other plastics are easier colonizable substrates than glass or metals (4). Rougher surfaces are preferred by microbes since shear forces are lower than on smooth surfaces and the overall area is bigger (1).

Fimbriae (i. e., exocellular, non flagellar appendances) are known to confer to the microbial cells hydrophobicity (5) and seem to overcome the initial repulsion between the cell and the substrate. Proteins are also play a role in attachment as the treatment of adsorbed cells with proteolytic enzymes is shown to cause their detachment (6,7).

The second phase of a biofilm formation is characterized by an increase in cell number by division and aggregation in microcolonies held together by bacterial slime. The first layer allows the subsequent colonization by other recruited organisms determining the final aspect of the consortium. Final specific composition, final cell amount and architecture of biofilms are determined by nutrient availability and hydrodynamic conditions. Besides creating availability of binding sites, the first colonizers provide for an available trophic source for other recruited organisms such as heterotrophic bacteria.

The third and last phase is characterized by cell detachment. Cells can actively move from the aggregates using their motile structures or be passively removed by erosion. Biofilms can detach collectively, by rolling or rippling across the substrate. There are three known processes of detachment: erosion (continuous removal of small biofilm parts), sloughing (rapid and massive removal) and abrasion (8,9).

The associations of microbes has ecological meaning. The microcolony can be defined as the 'core' unit of a biofilm. In its proximity ideal conditions in terms of trophic resources, quorum sensing and gene exchange (conjugation) are established and, since microcolonies are constituted by different species with different capabilities, cycles of various nutrients such as nitrogen, sulfur and carbon can take place.

Within a biofilm, antagonistic relationships can establish between microcolonies following inter-colony competition, as reported by James et al. (10) though in many cases a coexistence in stable communities is reached. The cohesion of cells can bring many advantages to every single strain as many processes are facilitated (e. g. many substances are more easily biodegraded, especially pollutants) and they can find ideal conditions to thrive. The spatial arrangement depends on the gradients of oxygen, substrate an pH value so that a large number of species can insediate in habitats otherwise not easily colonizable individually (11, 12).

1.1.1 Techniques for studing biofilms .A detailed observation of the microorganisms forming biofilms was only possible after the use of the electron microscope, which allowed higher resolution than the light microscope. A scanning electron microscope (SEM) was used by Jones and collaborators (13) at the end of the sixties to observe the thickness of microbial films upon the filters of a wastewater treatment plant. Basing on cell morphology they were able to distinguish a variety of species composing the consortia.

Throughout the seventies on, many studies have been carried out in order to understand the features ruling biofilms formation through attachment to living and non-living substrates and, most of all, to increase the knowledge on those aspects more relevant for human health. Since biofilms formation concerns different fields, studies have spread in width from industrial to ecological and medical environments. In 1978 Costerton elaborated a theory explaining the ecological meaning and

advantages of cell attachment to surfaces, starting from data obtained from dental plaque and microbial communities in mountain streams (14), while five years earlier Characklis made observations of biofilms in industrial water systems, noticing their resistance to disinfectants as chlorine (15). Through the years several optical methods have been developed to observe undisturbed biofilms, though in some cases these methods are limited by biofilm thickness. In the eighties and nineties the use of confocal laser microscope (CLSM) allowed to observe the ultrastructure of communities. CLSM allows the observation of living hydrated biofilms providing informations on the tridimensional architecture and components, using dyes (to evidence DNA, EPS glycoconjugates) or using the auto-florescence in the case of green microalgae. Scanning transmission X-ray microscopy (STXRM) can be used to observe fully-hydrated biofilms as x-rays are able to penetrate water molecules and also map metals. As demonstrated by Lawrence et al. STXRM can be combined to CLSM and transmission electron microscope (TEM) to have a detailed description of the structure and the component of a biofilm (16). Atomic force spectroscopy (AFM), Attenuated Total Reflection Infrared spectroscopy (ATR-IR) and Nuclear Magnetic Resonance (NMR) are also used to get precise information at a molecular level with an higher precision.

Genetic investigations led to understand the genetic dynamics ongoing inside developed biofilms An high number of genes showed to change their expression when passing from a planktonic form to an aggregate form, regulating the growth rate and consequently the phenotype of the consortium. Up- and down-regulation of specific genes is triggered through *Quorum sensing* , which defines a complex of signals representing the communication between cells (17).

Scientific investigations on a biofilm can be direct to its components, its structure/architecture or its internal physiological and biochemical processes.

Studies have been carried out in natural environments or in laboratory conditions where devices were designed in order to grow biofilms under controlled parameters (18). In laboratory conditions biofilms are investigated in two different ways: to a physiological level and to a genetical level: in the first case, using microsensors, a wide range of parameters on communities such as nutrient consumption and mass transport rate can be evaluated; in the second case, beside a qualitative analysis on the species present, reconstruction of genomes and individuation of specific genes

can be triggered using PCR, pulse field gel electrophoresis (PFGE), denaturating gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH).

1.1.2 Extracellular Biosynthetic polymers. Extracellular biosynthetic polymers, which constitute from 50% to 90% of the total organic matter of the biofilm (19), usually called EPS, are defined in literature as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates" (20) or are defined as "organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion) (21).

It appears as an high hydrated mucous slime lying outside (by definition) the pseudomurein layer of the archaea, the cell wall of gram+ and the outer membrane of gram-. EPS have been defined as "the house of biofilm cells" (22) as they can condition the life of the organisms dwelling within by affecting porosity, density, water content, charge, sorption properties, hydrophobicity and mechanical stability of the bio-layer (see table 2), playing a major role in conferring biofilms a big versatility towards external conditions.

Table 2 *Functionality and role of EPS components (22,23)*

Effect of EPS component	***Nature of EPS componentt***	***Role in biofilm***
Constructive	*Neutral polysaccharide; amyloids*	*Structural component*
Sorptive	*Charged or hydrophobic polysaccharides*	*Ion exchange, sorption*
Active	*Extracellular enzymes*	*Polymer degradation*
Informative	*Lectins, nucleic acids*	*Specificity, recognition, genetic information, structure*
Redox active	*Bacterial refractory polymers*	*Electron donor or acceptor?*
Nutritive	*Various polymers*	*Source of C,N, P*

First studies on its nature were made using a dye called Ruthenium red coupled with osmium tetroxide (13) which allowed to appreciate that the material surrounding cells was, as a matter of fact, composed by polysaccharides. The term EPS in some cases can be misleading since sometimes it represents an abbreviation for "Extracellular Polysaccharides", "Exopolysaccharides" and "Exopolymers". It also stands for "Extracellular Polymeric Substances" and, since they are shown to be composed by a variety of molecules of different origin, this latter definition results more enlightening.

EPS are composed by polysaccharides, proteins, nucleic acids (19, 24, 25) phospholipids (26, 27) and some researches describe humic acids as well, as a component (28; 29). Most of the sources report polysaccharides as the primary components (1) though others report them being in some cases a minor component (25).

EPS can be the result of different processes including secretion, cell lysis or adsorption from the environment. Polysaccharides and proteins are secreted by living cells and are dispersed into the surrounding medium, as described by some authors (30, 31); it is discussed whether DNA is secreted outside the cells or released through the cell envelopes following an increase in permeability (32). Lipopolysaccharides (LPS), which are cellular components in gram- bacteria, are released through the formation of blebs (outer-membrane derived vescicles) (33, 34). Other intracellular molecules such as poly-β-hydroxyalcanoates, peptidoglycan or glycogen, though not regarded as EPS, can enrich the outer matrix following cell lysis.

Enzymatic and/or degradation processes produce the variability of EPS composition in time and space. Humic substances, which are present in soil and water biolayers, are thought to be the result of enzymatic degradation coupled with repolymerization (35).

Polysaccharides can be structured in three different ways around a single cell or a cell group: as a 'sheath', consisting in a thin electron dense layer; as a 'capsule', which is a thin layer associated with cell surface; as 'slime' which is a mucilaginous material not respecting the shape of the cell. These structures are encompassed under the term "glycocalix", which possibly include also S-layers, composed of proteins and glycoproteins (36).

The term slime is used to indicate a non condensed layer loosely associated though not dissolved. The dissolved portion of polysaccharides in literature is often called ‘colloidal’. The slime appears dispersed around the cell and is shed in the surrounding medium.

Capsular polymers, which are firmly attached to cell surface, are associated with non covalent bonds or with covalent bonds with phospholipids or lipid-A molecules (37).

In some cases such as those of filamentous cyanobacteria or bacteria of the genera *Leptothrix* and *Sphaerotilus* the polysaccharide is organized as a sheath surrounding cell, appearing structured as a capsule in unicellular strains (27).

Microbial ‘mucilage’ is another term related to EPS, which is composed by aggregates of colloidal macromolecules which can arise to massive accumulation, especially in aquatic ecosystems. The consistence of the mucous envelope is also related to the level of jellification which in turn is determined by covalent bonds (e.g. sulfur bridges in proteins) and ionic bonds stabilized by bivalent cations. Where there’s major chemical stabilization the envelope is more rigid and compact and more associated with cells. Therefore somewhere in literature EPS can be found differentiated for their association with cell walls which can be appreciated after the centrifugation: the polymers found in the supernatant (colloids, slime and soluble macromolecules) are defined ‘Soluble EPS’ while the polymers bound to the pellet (sheaths, capsules, loosely bound polymers, attached organic materials) are defined ‘Bound EPS’ (figure 1 (38)). This simplify the distinction between the fractions bypassing the need to account for the fact that most of the chemical state of the matrix depends on environmental factors such as available ions and pH and thus it is susceptible of variations.

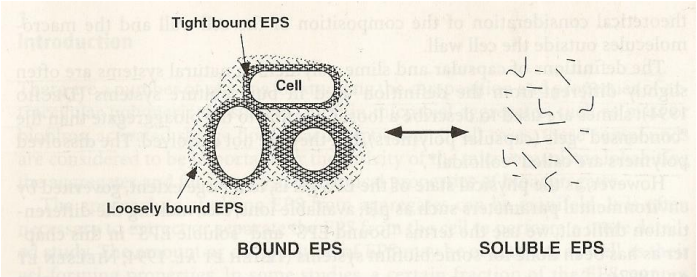


Fig. 1 *Schematization of extracellular polysaccharides embedding cells and soil particles (38).*

Exopolysaccharides have molecular masses varying between 500 and 2000 kDa (39). Though exopolysaccharides can be neutral, they more frequently show charged molecules such as carboxyls and uronic acids or groups such as phosphate, sulfate, glycerate, pyruvate or succinate (40) which confer negative charge, or amino-sugars, which confer positive charge.

While an high number of polysaccharides is soluble in water or in dilute saline solutions, some other are not soluble and tend to form very stable gels. These polymers are structurally characterized by a backbone containing 1,3 and 1,4 linkages, presenting α- or β- configuration and are those who mostly confer stability to the biofilm structure.

While in some cases polysaccharides are homopolymers, in other cases they can be qualitatively complex in terms of monomers and linkages and often form associations with other molecules contained in the matrix (e. g. proteins and lipids) influencing the viscosity of the medium and other chemical properties. *Escherichia coli* and *Enterobacter cloacae* produce colonic acid, which is composed by a repeating unit of a hexasaccharide made up of four sugars with the presence of acetyl and pyruvate groups (41). Pereira et al. (42) reported that in the phylum cyanobacteria 8% of the studied strains (accounting as far as 2009) secrete polysaccharides composed by more than 10 monomers.

Polysaccharides can be also involved in electrostatic interactions with ions such as calcium, magnesium and iron thus influencing the compactness of the saccharidic envelope and flocs formation as divalent ions (especially calcium) act like bridges collating polymeric strands *via* ionic bonds. These interactions stabilize the matrix in an ordinate structure as well as providing for the availability of ions for the biochemical activities of the cells. The ionic complement of any biofilm is normally correlated to the nature of the sugars and to the configuration of the polymeric structure, thus cations are not homogenously distributed in the matrix as it is confirmed from observation from Thu and coll. (43) using synchrotron X-ray emission in alginates.

Many studies have questioned on the function and the ecological significance of EPS synthesis in biofilms.

General functions first attributed by researchers concerned their structural role and their contribute to the adherence to the substrates. Moreover their role in the

establishment of infections and their protection against environmental factors were also underlined. This latter subject was discussed by LeChevalier et al. (44) and Foley and Gilbert (45) who observed that cells associated in biofilms bear higher values of certain biocides mostly thanks to the presence of the matrix as a barrier. Other proposed roles include cell-cell recognition, scavenging of xenobiotics, and detoxification from toxic metals of the environments as well as accumulation of secreted enzymes (42, 46, 47).

From an ecological point of view EPS allow cells to aggregate together forming synergistic microconsortia in which every specie takes advantage from the cohabitation. Furthermore EPS works as a sequestering agent of nutrients from the aqueous phase thus making them available for the microorganisms.

Genetic material can be easily conserved and, as the organisms can move and take it up, gene exchange is promoted. Horizontal gene transfer (conjugation, transduction and natural transformation) is facilitated by the proximity of aggregated cells while DNA is conserved within EPS.

Quorum sensing is also facilitated still by the proximity of the cells and by the accumulation of molecules such as N-acyl-L-Homoserine Lactones (AHLs) in the matrix.

1.1.3 EPS analysis. While the first studies on EPS were performed on pure cultures (pure culture philosophy), nowadays the majority of microbiologists work with EPS matrices coming from complex microbial communities (48).

The analysis on EPS can be carried out using “destructive” and “non-destructive” techniques. Extraction, sugar analysis, linkage analysis, sequencing and electron microscopy, among the destructive, and infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) and confocal laser scanning microscopy (CLSM), among the non-destructive, are the most frequently utilized techniques.

The extraction is the starting point for further chemical or structural analysis.

No universal extraction method exists and it has to be evaluated in the single case as it mostly depends on the type of interactions that keeps polysaccharides together, which are represented by van der Waals forces, hydrogen bonds (mostly as disulfide bonds in glycoproteins) and hydrophobic interactions. Furthermore it also depends

on the aim of the extraction as in some cases extraction has to be not aggressive as no cell lysis is wanted.

Physical methods include centrifugation, shaking, stirring and variation in temperature. Centrifugation is normally used to divide the soluble fraction (loosely bound polysaccharides or slime) from the biomass. Relying on this method tighter bound EPS are rarely extracted (25, 49). Often physical methods are coupled with chemical methods which include a wide range of extractants which break or loosen linkages thus facilitating the recovery of the polymeric material.

Alkaline treatments performed using NaOH(1-9 N) can increase the solubility of the sugars after creating a strong repulsion within the EPS gel, though sometimes it is reported to cause cell lysis (50) and to break disulfide bounds (51). Removal of divalent cations such as Ca^{2+} and Mg^{2+}using resins (Dowex) or EDTA solutions can loosen the EPS stability by breaking the ion bridges between the strands, though some care must be taken as it can lead to the destabilization of cell walls causing leakage.

Saline solution treatments have been also used (52, 53, 54) coupling them in some cases with ion exchange compounds (55).

Cell lysis control is important to be considered when performing an extraction, though there is no universal method to determine the extent. Determination of DNA, proteins and lipids in the extract was proposed but since these compounds are already present in the cell environment it is difficult to use their concentration as an indicator. ATP was also suggested as an indicator though it does not give good accuracy (56). Another indicator suggesting cell rupture is the enzyme glucose-6-phosphate dehydrogenase (G6PDPH) (57) which it is only intracellular, though small traces may still be found in the environment following natural cell lysis.

1.2 Biofilms on monuments

Besides representing a treat in medical and industrial field as already mentioned, biofilms can also represent a menace for the preservation of cultural heritage, especially lithic artworks. Both historic and modern buildings can be affected by microbial colonization leading to irreversible chemical and physical biodegradation. Chemical biodegradation is caused by secretion of acidic metabolites and siderophores, while physical biodegradation is caused by penetration of the organisms into the substrate (58, 59).

Microbial colonization depends on environmental factors such as water availability, pH, trophic sources and petrologic parameters (such as porosity and permeability of the rocks) (60, 61).

Prototrophs, cyanobacteria and microalgae, are considered to be the primary colonizers (62) and represent the most frequently recurring groups in stone biofilms. They build a visible protective biofilm enriched with inorganic and organic biomass which represents a trophic base for heterotrophic organisms including bacteria, fungi and protozoa. Chemolithoautotrophs are frequently observed and contribute in the stone weathering as they secrete acids such as nitrous acid (for instance *Nitrosomonas* spp.), nitric acid (for istance *Nitrobacter* spp.) or sulfuric acid (for istance *Acidothiobacillus* spp.) which contribute to stone decay. Biogenic acids are considered the major damaging agents and fungi are considered to be the primary organisms causing the biocorrosion of the rocks (63).

Stone colonizing cyanobacteria can be epilithic or endolithic, and lead to the biogenic staining of the stone. Some investigations have reported them as the primary contributors in stone decay as they excrete corrosive substances (64), and some are capable of acid degradation (65). They hold a key role as enhancers in biofilm formation as they are adapted to survive repeated drying and rehydration cycles occurring on exposed environments (66) and produce large amounts of EPS, creating a thick envelope conferring stability and cohesion between cell aggregates.

Chasmoendolithic cyanobacteria are adapted to grow inside stone fissures and then exerting pressure from within following drying and swelling cycles. The pore enlargement allows further microbial inner colonization as well as dust and pollen to

enter (64) leading to pore distribution alteration and changes in moisture circulation patterns.

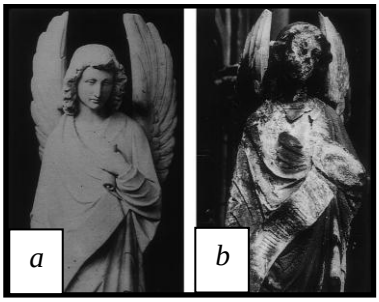


Fig. 2 *Results of bioweathering on an angel statue at the "Peters"-Portal on the cathedral of Cologne (Germany). Comparison of before (a) and after (b) the biodeterioration processes (71).*

High-porosity rocks (from 14 vol % with an average pore radius between 1 and 10µm) allows the penetration of moisture, leading the way to microbial contamination in the first 3-5 cm depth. Small-pore stones are more likely to enhance microbial contamination due to their higher water retention (67, 68).

On monuments microbial contamination is manifested at first as a discoloration of the stone surface following the deposition of organic pigments giving aesthetically unacceptable appearance (69, 70). Major pigments are chlorophyll by cyanobacteria and green microalgae, and mineralized products such as phaeophytin, phycobiliproteins and carotenoids.

Stone stains can be divided in three classes (71):

I. Black stains, caused by melanins, melanoidins, producs of chlorophyll degradation, iron and manganese minerals;
II. Green and greenish stains, caused by photosynthetic pigments;
III. Yellow-orange-brownish stains, caused by carotenes and carotenoids and degradation products from phycobiliproteins;
IV. Bright, orange, pink and red stains, caused by degradation of chemoorganotrophic bacteria and degradation products of cyanobacteria and green microalgae with iron enrichment.

The result of microbial activity is the formation of decay forms so called *patina* and *scialbatura*. The first arises from chemical and physical weathering, the latter is the result of deposition calcium oxalate mono hydrate and calcium dehydrate as a result of bio-corrosion (72, 73). In all the cases, if no countermeasure is taken, biological activity is likely to bring to the irremediably loss of the heritage or at least of its original shape (figure 2 (71)).

From a chemical point of view, microbial bioweathering can be divided in two possible mechanisms (74): soluble and insoluble. The former includes acid reactions (mainly carried on by chemolitotrophic bacteria), enzymatic and non-enzymatic processes; the latter includes oxidation, reduction of sulfur complexes and degradation of chelates.

The action of non- or weakly complexing acids on the stone is summarized by the formula by (75) as reported by (71):

$$(\text{mineral})^{-}M^{+} + H^{+}R^{-} \leftrightarrow H^{+}\,(\text{mineral})^{-} + M^{+}R^{-}$$

Where $R^{-}=NO_3^{-}$; R_1COO^{-}, HCO_3^{-}, SO_4^{2-}

Chemoorganotrophic bacteria and fungi release chelating organic compounds including ossalic acid, citric acid and oxalacetic acid or can oxidize cations such as Fe^{2+} and Mn^{2+}(64, 76).

1.2.1 Cation sequestering from the substrate. Another chemical cause of the decay process is represented by cation transfer from the substrate to microbial cells. This can happen in two ways: by intervention of siderophores or by active ion-uptake (77, 78); in both ways final immobilization follows redox reactions enhanced by cyanobacteria-produced oxygen (79) or, in the case of iron and manganese, by the activity of heterotrophic organisms (80), leading to oxide accumulation outside the cells.

Extracellular polymeric substances can bind metal cations such as calcium, magnesium and iron (81), depaupering the stone. In presence of acidic groups (carboxylic acid, sulfate or phosphate groups) Ca^{2+} form bidentate bridges between

adjacent polymeric strands thus remaining immobilized (82). Calcium binding capacity of EPS varies between 50 mg Ca/g and 180 mg/g EPS (83). Mg^{2+} also strongly binds to EPS so that it becomes calcium competitor in some environments.

The activity of EPS as a nucleation site for calcium and magnesium inhibits calcite precipitation until no binding sites remain and/or sugars are decomposed or altered through chemical and/or biological activity. Thereafter HCO_3^- and Ca^{2+}or $Mg^{2+}$are released and can combine (84) determining encrustations.

Besides disfiguring the stone, this processes leads to permanent changes in the crystalline structure, leaving the substrate more fragile and susceptible to erosion.

Ion sequestering by EPS has the role of making available ions for the cells as well as of cementing the biofilm. Multivalent cations mediate the stabilization of the saccharidic matrix acting like bridges that cross-link the polymeric strands. In this way the cohesiveness of the envelope is dependent on the quantity of produced exopolysaccharides but also on the ion concentration.

EPS are also capable of binding iron ions which are important for the photosynthetic apparatus. Brown et al. (85) reported three functions served by extracellular iron accumulation for iron-tolerant cyanobacteria: to produce reserves of iron, to shield the cells against UV rays and to decrease the chemical potential of active iron. The complexes EPS-iron stays even after cell death and results in the preservation of microfossils.

1.2.2 UV-screening pigments. Many cyanobacterial strains are capable of synthesizing UV screening pigments, which give them the capability to withstand high solar irradiations. The presence of scytonemin and mycosporine-like aminoacid substances (MAAs) allows cyanobacteria to shield themselves, and other microbial cells devoid of screening pigments, against harmful UV-rays and thus to grow on exposed-surfaces like rooftops or external walls. Scytonemin is an indol- alkaloid dimeric molecule (MW 544 Da) located in the bacterial sheath and adsorbing in the UVA and visible range(315-450nm), in the UVB from 280 to 320nm and in the UVC with a peak at 250nm (86) and show an *in vivo* maximum at 370nm. It is inducible in living cells by UV-irradiance, most notably in the UVA and UVB wavelengths. It is proved that common levels of scytonemin in bacterial sheath is capable to prevent 85-90% UV-A radiations from entering the cells. MAAs are a family of molecules

constituted by a substituted cyclohexanone linked with an aminoacid or its amino alcohol. They have been characterized in fungi, eukaryotic algae, corals and starfishes, and are a family of colourless, water-soluble compounds with an adsorption maximum between 310 and 334 nm showing a characteristic single adsorption band between 230 and 400nm (87, 88). Unlike scytonemin MAAs are mainly intracellular, though they can be found also extracellularly linked to oligosaccharide compounds. Their synthesis can be elicited by ultraviolet radiation as well as osmotic stresses. It was also reported their role in contributing to cell turgor (89,90), being an osmotically active solute.

1.3 Biological Soil Crusts

Biological Soil Crust (BSC) are highly specialized communities of macro and microorganisms ubiquitous of arid and semiarid desert landscapes of the world. They are structured as an uneven carpet from 1 to 10 cm height. Their importance has been recognized in recent years for playing a crucial role in biogeochemical and geophysical processes of those landscapes, as they play both structural roles for the soil and provide for nutrient, moisture and fixed-nitrogen availability, representing a potential tool for contrasting the desertification processes.

BSC can be used as indicators for soil health and indicator of abiotic factors like the presence of calcareous soil.

Unlike vascular plants, that are the historic indicators of environmental health, BSC are not greatly influenced by short-term conditions, so that they represent long-term indicators for a given site.

Cyanobacteria are ubiquitous components of BSC, except in low-pH environments, and they are considered to be the first colonizers of bare desertic soils and the initiators of BSC growth during episodic events of available moisture.

1.3.1 Biological Soil Crusts components. BSC are associations of phototrophs (cyanobacteria, green microalgae), non-vascular plants (bryophytes), lichens, microfungi (free-living organisms and micorrizal associations) and other bacteria

such as proteobacteria. Filamentous cyanobacteria and fungal hyphae glue loose soil particles together and create a stable structure resisting to erosion and allowing further colonization.

Components of BSC are referred to as "poikilohydric" as they are all capable of drying out and suspend respiration to adapt to drought periods and photosynthesis starts quickly few minutes after wetting. Furthermore most of them are able to reach out for humidity and soil moisture to equilibrate their water content.

One of the most common cyanobacteria inhabiting BSC is the non-heterocystous *Microcoleus vaginatus* that appears as a cluster of filaments surrounded by polysaccharidic envelope (sheath) when magnified (figure 3 (91)). *Microcoleus* lacks UV-protective pigments thus inhabits the first 1-4 mm below the soil. It is able to move up reaching and covering the soil surface during wet periods with low solar irradiation and then move down again during drought times. Unicellular cyanobacteria (*Nostoc*, *Scytonema*) colonize later and live on the soil or just below the surface; they appear generally small and blackish.

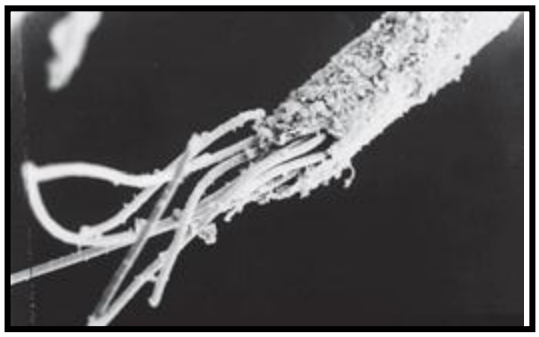


Fig. 3. *Filaments of* Microcoleus vaginatus *surrounded by mucous sheath (91).*

The high concentration of cyanobacteria determines soil roughness, derived by soil particle binding together and by frost-heaving and erosion like processes (92). Later on, if it is allowed by climatic conditions, lichens and mosses colonize and live covering the soil surface.

Bryophites are non-vascular plants including mosses and liverworts. Both these life forms often (always, in the case of liverworts) reproduce asexually in arid

environments, mosses throughout spore capsules and liverworts by gemmation and sporulation.

Lichens are association of fungi and green microalgae or cyanobacteria. They can completely cover soil surface organized in form of leaves. They can reproduce sexually or asexually.

Lichens and bryophytes have anchoring structures (rhizoptae, rhizinae and rizomorphs) that penetrate 14mm into the soil fixing and stabilizing the mature crust (93).

Bacteria in BSC can be autotrophic or heterotrophic. Some collaborate in nitrogen fixation while others lead decomposition processes together with free-living fungi.

1.3.2 Different BSC types. BSC can be classified by their aspect:

- Smooth crusts, which recur in hyper arid drylands (Sahara desert) where soil never freeze and Potential Evapo Transpiration (PET) is very high;
- Rugose crusts, recurring in drylands where soil does not freeze but there is a lower PET (Sonoran, Mojave, Australian deserts)
- Pinnacled crusts, recurring in cool deserts where soil freezes and there is a lower PET than in hot deserts;
- Rolling crusts, recurring in colder regions with still lower PET (northern Great Basin). These crusts are dominated by lichens and mosses.

These categories are useful when discussing water infiltration in the soil (see later).

BSC can be classified also for their morphological groups, depending on the dominant organism:

- Cyanobacterial crusts, which appear dark colored or black;
- Green microalgae crusts (though not always visible), which appear green when wet;
- Moss crusts, which appear as a carpet with patches of green, gold, black;
- Lichen crusts, which can be identified by their diversity of shape and color depending also on the lichen morphology (crustose, gelatinous, squamulose, foliose, fruticose).

BSC have only recently been recognized in their importance for the ecology or arid environments. They are effective in reducing wind and water erosion (94) especially moss crusts (95). EPS by cyanobacteria and green microalgae enhance soil stability

binding particles together in combination with moss rhizines and lichens, allowing soil stabilization and avoiding soil loss. Furthermore they reverse a big quantity of nutritive substances in the soil allowing fertilization.

1.3.3 BSC and hydrological distribution. Another matter of study is about the influence that BSC may have on the water distribution in soils in extreme environments. These environments are characterized by water sinking areas and run-off areas, with the first becoming the productive patches. The shedding areas are instead poorer, with sparse vegetation and low nutrient content. This ecological equilibrium is kept also thanks to soil biological cover which interferes with water penetration.

It has been discussed in the last decades whether BSC have a role in affecting water distribution in the soil. In this sense, results in literature are ambiguous. Most of the authors report a decrease in water infiltration following microbial cover growth in the soil. This is due to the reduction of soil porosity and pore clogging of crustal organisms (96). Nonetheless some researchers associate crust growth to an increase in water infiltration (98, 99). What is straight is that many factor concur in determining crusts relationships with water, thus implying more complex evaluations to be done (see chapter 4).

2. PhD Experimental work

2.1 Aim of the thesis.

The objective of this thesis was to investigate those adaptation strategies allowing the development of microbial life in extreme environments, in particular on building stone surfaces and arid environments.

The research focused especially on the features of EPS of microbial origin and their contribution to microbial spread.

Considering these two kind of environments, in both cases four main constraints condition microbial colonization:

i. Exposition to erosive agents, most notably wind, which creates the need of a stabilizing agent, represented by EPS;
ii. Exposition to ultraviolet solar radiations, which creates the need to prevent cell damages;
iii. Dryness, which creates the need to handle the moisture coming from rainfalls, from atmospheric humidity and from the morning dew.
iv. Lack of nourishment, which creates the need to maintain the availability of minerals, sugars and fixed nitrogen.

To analyze these different aspects , several activities have been carried out.

In particular, working on lithic substrates colonizing cyanobacterial biofilms:

I. The Released Polysaccharides (RPS) from axenic cultures of five cyanobacterial strains isolated from Indian stone biofilms were extracted and characterized investigating both qualitative (number of different sugars) and quantitative features (relative abundance of each monomer);
II. The capacity of the isolated strains to remove bivalent cations was assayed, in order to estimate their capacity to supply cells with calcium, magnesium and iron;
III. The adaptation to UV irradiation of axenic cultures was evaluated, also investigating the capability of the isolated strains to synthesize UV-protective pigments.

Working on Biological Soil Crusts from North American deserts:

I. A method for the quantification of the total carbohydrates (both soluble and tightly bound to particles and cells) and of high molecular weight carbohydrates constituting the polymeric matrix of the crusts was optimized and standardized;
II. A new method for the extraction of the whole crust polysaccharidic envelope, capable of leaving the structure of the crust intact and allowing other subsequent assays on the sample, was developed;
III. Extracted polysaccharides were purified for subsequent qualitative and quantitative analysis;
IV. A methods to measure hydraulic conductivity was standardized in order to investigate the possible correlations between water penetration in the soil and the presence of exopolysaccharides.

2.2 Polysaccharides extraction, quantification, purification and analysis.

2.2.1 RPS extraction from axenic cultures. From single cultures RPS were separated by centrifugation from the cells to collect polysaccharides in the supernatant. This procedure does not allow to obtain all the extracellular polysaccharides but only the fraction in a lower jellification state (slime or soluble carbohydrates) while tightly bound polysaccharides remains with the cells. Nonetheless this fraction is representative of the biofilm envelope, as it represent the fraction which is shedded and dispersed and ends up embedding other organisms and sediments. RPS must be purified prior the analysis (paragraph 2.2.1) as free DNA, proteins and lipids are normally present in the cyanobacterial envelope. The use of extractants with the aim to extract cellular sheath is reported in literature, though no universal method exists and the risk of cell rupture is higher when dealing with pure cultures.

2.2.2 Extraction and quantification of soil carbohydrates. A method allowing to quantify EPS in the soil was optimized in order to approach a study on microbiotic crusts of desert environments. For this purpose an extraction and a subsequent quantification were needed. A correct method for the extraction of extracellular carbohydrates must be capable to avoid cell damages, as eventual leakages of intracellular carbohydrates could interfere with carbohydrate quantification.

The method proposed by Underwood et al. (54) was optimized for the aim of the study. Ethylenediaminetetraacetic acid (EDTA) was used to remove EPS more tightly associated with the cells and with sediment particles. EDTA is a chelating agent and its effectiveness is related to its capacity to bind cations (mostly Ca^{2+} and Mg^{2+}) which stabilize the polymeric strands.

The use of this quantification method allows to obtain a range of informations in a relatively short time thus to run simultaneously a large number of replicates. The extraction procedure is carried on with small quantities of sediment (100 mg) so that small crust fragments collected randomly from the samples are already a satisfactory and representative quantities. Fragments are gently homogenized in a mortar, treated with 0,1 M Na_2EDTA for 15' at 20°C and then centrifuged at 3600 x *g*.

The incidence of cell rupture after EDTA extraction was discussed by Underwood et al. (54) whom found no evidence of it in their results, concluding that it was absent or minimal.

Extracts are precipitated in 70% ethanol (final concentration) and the washed precipitate (consisting of high molecular weight sugars) is termed EPS, according to the definition proposed by Decho (100).

The quantification of extracellular carbohydrates in the extract was done using Phenol Sulfuric acid assay, according to Dubois et al, (101).

The assay is sensitive to a wide range of carbohydrates including sugars, methylated sugars, neutral and acidic polysaccharides which are constituents of EPS. For the quantification, 1ml of extract is treated with 1ml of 5% Phenol followed by 5ml of pure Sulfuric acid and stirred. After 10' they are cooled up in water for 15' and then spectrophotometrically measured at 480 nm.

The absorbance measured at 480 nm is in direct correlation with the concentration of carbohydrates. The method described by De Brower and Stal (102) was also considered. The authors utilized a method of extraction aimed at separating the different fractions constituting the polysaccharidic matrix: the soluble fraction, which is weakly bound to cells and sediments and is dispersed in the soil, that can be recovered simply treating with water and the tightly bound fraction, which needs an extractant as it is in an higher jellification state. Also in this case, 0.1M EDTA was used as extractant. They performed a first 60' distilled water extraction at 30°C. The extracts were centrifuged for 5' at 6000 x *g* and the supernatant (containing soluble

or weakly bound EPS) was stored frozen. Thereafter 0.1 M EDTA was added to the pellet and maintained for 16h at room temperature. The extracts (containing tightly bound EPS) were centrifuged for 5' at 6000 x *g* and the supernatans were stored frozen.

2.2.3 Optimization of soil carbohydrates extraction and quantification for North American Biological Soil Crusts. The extraction procedure was optimized on North American Biological Soil Crust (BSC) samples.

Four soils, corresponding to the four major north American deserts were chosen: one loamy sand sample from Colorado Plateau (CP 1090), one silt loam from Chihuauan desert (CD 788), one sandy loam from Sonoran desert (SD 692) and one loamy sand from Mojave desert (MD 854). Three steps were followed:

1st step. Extraction with water 60' to recover and quantify the 'immediately colloidal' fraction;

2nd step. Further treatment of the water extracted sample with EDTA 0.1M 15' to recover and quantify the 'bulk' fraction;

3rd step. Direct extraction with EDTA 0.1 M 15'.

Results are summarized in figure 4.

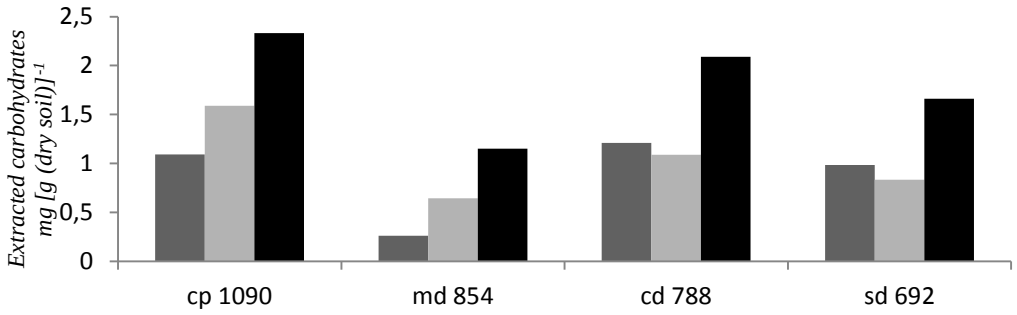


Fig. 4. *Extracted carbohydrates (expressed as mg [g dry soil]$^{-1}$): after extraction with deionized water (dark grey bars);, extraction with EDTA after extraction with deionized water (light grey bars); direct extraction with EDTA (black bars).*

Abbreviations: cp, Colorado Plateau; md, Mojave desert; cd, Chihuauan desert; sd, Sonoran desert.

Longer EDTA extraction times (up to 16h) were tested on the samples but no significant increase in the carbohydrate yield was observed, suggesting the immediate effectiveness of EDTA and confirming the data obtained by Underwood

et al. (54). The short time needed for the treatment allows to avoid cell leakage risks due to the chelating activity of the extractant that can destabilize cell walls.

The comparison between direct EDTA extraction and water extraction points out the notably higher carbohydrate yield obtained in the first case. This major effectiveness was probably due to the capacity of the extractant to remove both the bulk fraction and the soluble fraction, doubling the yield obtained with water only. Extracting directly with EDTA led to a cumulative amount that resulted close to the amount obtained extracting with water followed by subsequent EDTA addition, suggesting that in the first case both fractions, soluble and bound, were removed at the same time.

The absorbance of the extracts at 665 nm was registered with a spectrophotometer and both pellets and supernatant after centrifugations were investigated for the presence of lysed cells using a light microscope, finding no evidence of cell leakage.

Outcoming data suggested the effectiveness of EDTA in removing carbohydrates though no data on the carbohydrate amount remaining in the soil was available.

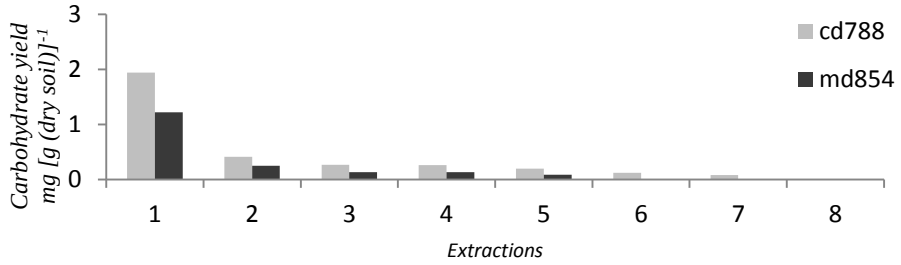


Fig. 5. *Repeated extractions of carbohydrates (expressed as mg [g dry soil]$^{-1}$) from Chihuauan desert and Mojave desert Biological Soil crusts.cd, Chihuauan desert; md, Mojave desert.*

Repetitive extractions were performed on Mojave and Chihuauan desert BSC sediments adding EDTA 0.1M. These two soils were chosen as they showed different texture: Mojave desert sample appeared mostly constituted of sand while in Chihuauan desert sample silt appeared conspicuous, thus probably needing a more energic extraction procedure.

Five extractions and seven extractions were needed respectively to lead to no more detectable carbohydrate amount in the samples (figure 5). In both cases the first extraction yielded a major amount of extracted carbohydrate than the further extraction, representing 60% of total extracted carbohydrates per gram of sediment in the case of Chihuauan desert and 70% carbohydrates over total in the case of Mojave desert. With three consecutive extractions, the carbohydrate yield comes to an 80% over total extracted carbohydrates in the case of Chihuauan desert and nearly 90% in the case of Mojave desert. In the case of Chihuauan desert 5^{th} and 6^{th} extractions yielded only small amounts of carbohydrates per gram of sediment while in the case of Mojave desert sample 5^{th} and 6^{th} extractions were almost ineffective indicating no residual carbohydrates.

The results indicates that from three to five repeated extractions are needed to be able to evaluate total extracellular polysaccharide content in the soil avoiding underestimation, suggesting the need a pre-screening before undergoing a soil under extraction treatment.

A quantification of high molecular weight carbohydrates is important to make a first polysaccharide size fraction evaluation in the samples. Extracts (containing soluble and tightly bound carbohydrates) can be divided in size fractions by precipitation in 70% (v/v final concentration) cold ethanol. The precipitate is then centrifuged at 6000x*g* and re-suspended in deionized water. A second precipitation is sometimes needed to completely remove low molecular weight polymers.

Percent EPS over total extractable carbohydrates was evaluated for all the samples and results showed that high molecular weight polymers (> 100kDa) represented from 25% to 40%.

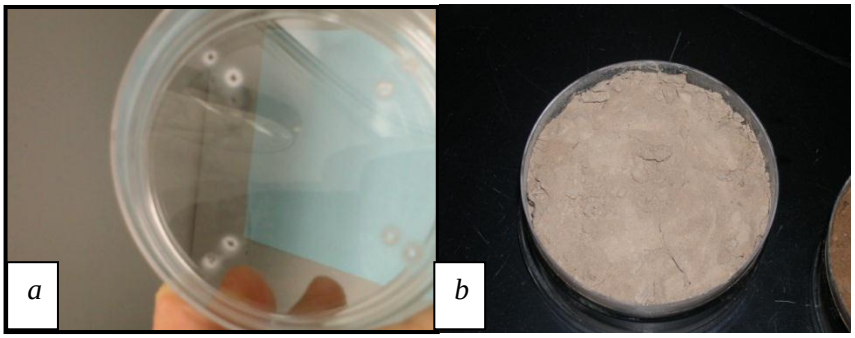
2.2.4 Non-destructive BSC matrix extraction. The quantification of total carbohydrate content of a sample is achieved through the omogenization of the crust, thus *de facto* limiting eventual further assays on the samples such as the measurement of the hydraulic conductivity (see paragraph 2.3.1).

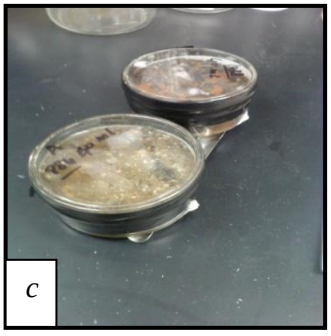
To extract extracellular polymeric substances and to maintain the structural integrity of the samples, a new method was developed during the second PhD year in collaboration with Prof Ferran Garcia Pichel, at the School of Life Sciences, Arizona State University, Tempe, AZ, USA.

The method is based on the suction of the matrix using a void pump after the matrix is destabilized with the use of EDTA.

For this purpose, the sample is placed in a plastic stand made up of two Petri dishes (see figure 6)

The sample is placed in the upper chamber which was previously holed with a hot tip (figure 6a) and treated with 20 ml 0.1M Na_2EDTA.





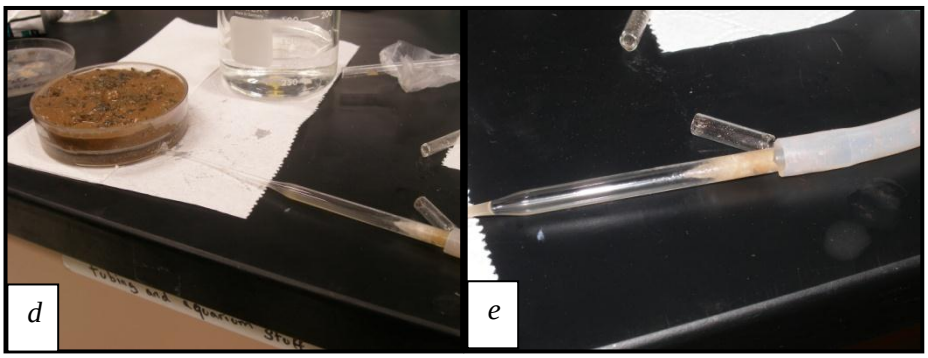


Fig. 6 (previous page). *Not- destructive matrix extraction method. After having done radial holes in the basement of the upper chamber (a) crust sample is placed (b) and treated with 0.1M EDTA for three hours with samples covered to avoid evaporation (c) Afterwards, a hole is done in the lowest chamber and a Pasteur pipette is inserted (d). The Pasteur pipette is connected to a void pump that creates a depression of -400/-600 mbar in the lowest chamber. This forces the EDTA solution to flow from the upper to the lowest chamber moving EPS which are collected in the lowest chamber and then aspired. The slime condensed due to the lowering of the temperature in the Pasteur becomes clearly visible (e).*

After three hours in the dark, another hole is practiced in the lowest chamber (figure 6d) and a Pasteur pipette is inserted. The void pump connected to the Pasteur creates a depression which forces EDTA solution to flow from the upper to the lower chamber moving EPS which are collected in the lowest chamber and then aspired.
Sample is finally washed three times, adding 20ml tap water each time, in order to remove the remaining EDTA and the residual extractable matrix particles.
The effectiveness of the treatment was proved measuring the total and high molecular weight carbohydrate concentrations (as EPS are mainly constituted by polysaccharides) before and after the treatment, using the method described in paragraph 2.1.2.
The decrease of carbohydrate content was coupled with visible changes in the samples. Although the method allows to maintain the integrity of the crust, changes in albedo were noticed (figure 7), suggesting a rearrangement of the structure due to the loss of the matrix.

.

Fig.7. *Comparison between non treated (a) and EDTA treated silty soil crust. The sample was dried in a ventilated oven at 32-33°C to remove humidity. A change in albedo is clearly observable after the extraction as white spots are alternated to dark spots where the color was uniformly dispersed. Surface curls are still present though less prominent while surface cracks are decreased in number.*

2.2.5 Purification of the extracted EPS. EPS from axenic cultures have to be purified from culture medium components (salts), proteins and pigments coming from cell rupture. Depending on the specificity of the sugar analysis, these molecules have to be removed to avoid possible interferences.

First cells are washed with deionized water and then evaporated at 50°C to concentrate the biomass. Polysaccharides are then precipitated using cold ethanol and the precipitate is dissolved in deionized water. The precipitation is repeated in total two times and it is useful to separate hydrophobic compounds from more hydrophilic ones. Hydrophobic compounds (including pigments) remain in the supernatant which is discarded.

Polysaccharides in solution are introduced in a dialysis membrane and dialyzed against deionized water for 24h to remove salts and then dialyzed against 0.1N NaCl in a ratio of 20:1 (20 NaCl solution to 1 of biomass) for 48h with 4 changes of dialysis solution.

A last dialysis is then performed against distilled water for enough time to be able to measure 1µSiemens of conductivity due to the low concentration of free ions.

After bringing the polysaccharide solution to pH=7 a spectrophotometric scan is performed in the range 190-350 nm (UVB and UVC) to evidence eventual peaks

indicating the presence of proteins. In case of a positive response, a protease must be added and then start over again from the dialysis against NaCl.

In case of polysaccharides extracted from BSC, EDTA remaining in the extracts must be removed as it can interfere with dry weight determination of the polysaccharides, with the protein determination and can obstruct any column in any chromatographic method, although it does not interfere with Phenol sulfuric acid assay (personal observations).

For this reason a 24-48h dialysis against distilled water is needed prior to proceed with the purification as described in this paragraph.

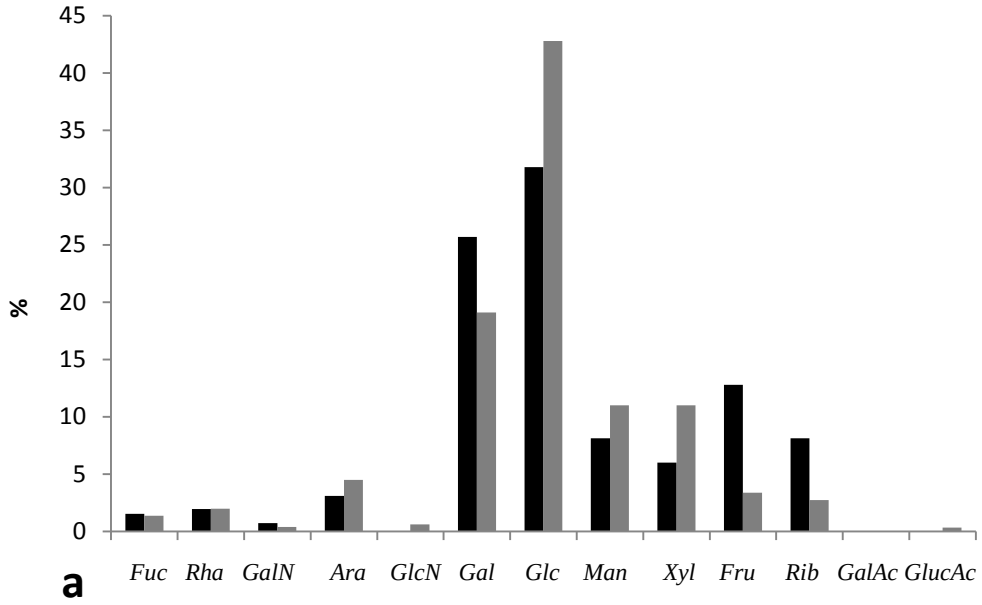
Before any analysis, polysaccharides are freezed and lyophilized.

2.2.6 Analysis of the monosaccharidic composition of EPS. Polysaccharides extracted from BSC and from lithic artworks biofilmswere analyzed for their monosaccharidic composition using a Dionex ICS-2500 ion chromatograph (Sunnyvale, Ca).EPS purified from other molecules and impurities have to be prepared for the injection. The first step is the hydrolysis, which is performed using Trifluoroacetic acid (TFA) which splits the polymers. 2N TFA is added to 5mg dried RPS at 120°C for a time between 45' to 120', sample are then cooled on ice and dried in a rotary evaporator. Before the injection samples are washed twice with MilliQ-grade water.

The chromatograph bears an ED50 pulsed amperometric detector using a gold working electrode (Dionex, Sunnyvale, Ca). A Carbopac PA1 4mm by 250mm column (Dionex, Sunnyvale, Ca) was used. The runs were performed at a flow rate of 1ml/min using three eluents: milliQ-grade water (S1), 0,488 Sodium hydroxide solution (S2) and Sodium acetate solution (S3). The first stage of the run is the injection time (from 0 to 7mins) with the eluent constituted by 84% S1, 15% S2 and 1% S3; the second stage, runs from 7^{th} to 13^{th} min with the eluent constituted by 50% S2 and 50% S3; the third stage, which is the column re-equilibration phase runs from the 13^{th} to the 30^{th} min with the eluent constituted by 84% S1, 15% S2 and 1% S3. The increase of NaOH and acetate determine the ideal conditions to reveal the presence of acidic sugars such as galacturonic acid and glucuronic acid that in fact are revealed in the second and third stage.

2.2.7 Monosaccharidic composition of polysaccharides from BSC. After developing an extraction method and an IEC analysis method it was possible to characterize extracted polysaccharides from a qualitative point of view. While for axenic cultures there's only one polysaccharide producer (see chapter 3), in biological soil crusts more organisms of the community secrete extracellular polymers, notably microfungi and green microalgae, in addition to cyanobacteria, thus expecting a more complex monosaccharidic composition. Polysaccharides extracted from Chihuauan 788 and Sonoran 692 BSC were extracted both in their dispersed fraction and in their fraction more adherent to cells and sediments. Samples were extracted first with deionized water for 60' and then with EDTA 0.1M 15'. Both fractions were processed for purification and hydrolysis before being analyzed by Ion exchange chromatography.

Results are summarized in figure 8:



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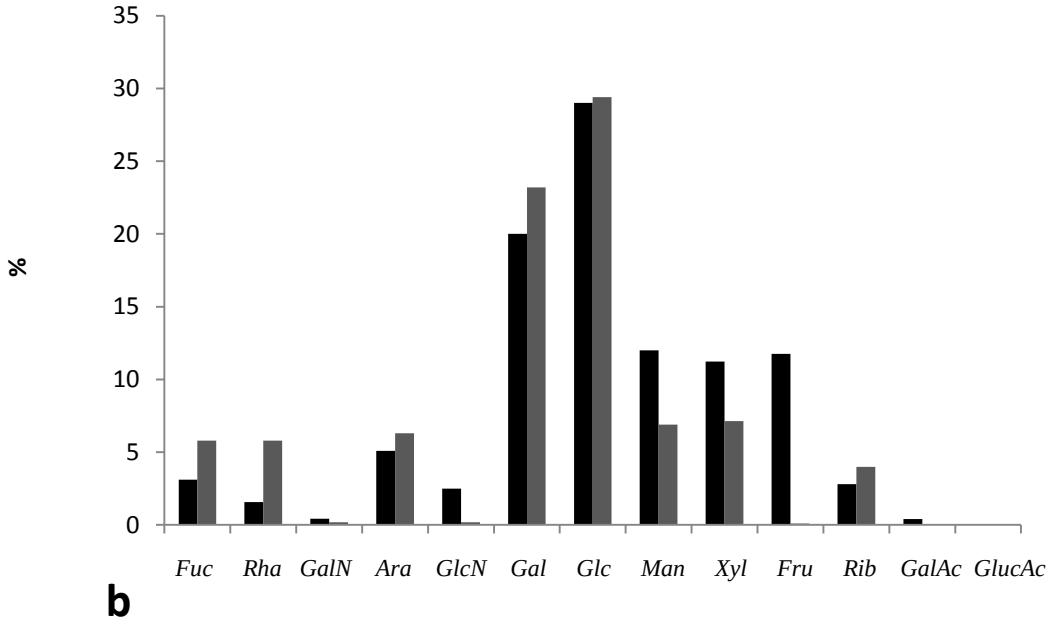


Fig.8. *Monosaccharidic relative abundances (expressed as % moles over total moles) of polysaccharides purified from extracted EPS from BSC collected in Chihuauan desert (a)(previous page) and Sonoran desert (b) after a first deionized water extraction (black bars) and subsequent EDTA extraction (grey bars) on the same sample*. *Fuc*,fucose; *Rha*, rhamnose; *GalN*, galactosamine; *Ara*, arabinose; *GlucN*, glucosamine; *Gal*, galactose; *Glc*, glucose; *Man*, mannose; *Xyl*, xylose; *Fru*, fructose; *rib*, ribose; *GalAc*, galacturonic acid; *GlucAc*, glucuronic acid.

The results show that with this method it is possible to extract both fractions and observe the relative abundances in their monosaccharidic composition. Variations in representativeness can be observed almost for every sugar comparing the two fractions and this might be the cause of a major or minor cohesiveness of the matrix. The fraction more adherent and hard to remove has an higher jellification state that is enhanced by ionic forces and covalent bonds (paragraph 1.1.2). In one case the use of EDTA helped recovering traces of glucuronic acid (figure 6a) which, as an acidic polysaccharide, is involved in cation bridging between the polysaccharidic strands, while in the other case (figure 6b) a first water extraction resulted sufficient to evidence traces of galacturonic acid, suggesting its presence in the slime fraction.

2.3 Hydraulic conductivity

Water distribution in the soils is related to their permeability, which in turn relies on a bunch of different factors. The texture surely plays a key role as compactness and the presence of cracks on the surface can affect water penetration.
Since BSC have been widely investigated for their possible role in water infiltration (thus having an important ecological role) a method to measure hydraulic conductivity (HC) in field samples was optimized and standardized and a possible correlation between HC and the presence of EPS was evaluated (chapter 4).
Soil permeability or HC in a saturated porous medium is described with Darcy's law expressed as:

$$q = -K\, dH\, [dZ]^{-1}$$

where q is the infiltration velocity, Z indicate the direction of the flow (upper is positive), K is the HC and H is the hydraulic load or piezometric load which accounts for height per weight unit and pressure energy per weight unit.
K is related to soil texture and varies from 10^{-1}-10^{2}cm/s in the gravel to 10^{-9} 10^{-5} cm/s in the clay. In saturated media water flow is affected by gravity and viscosity.
If Darcy's law is applied in a non saturated porous medium HC is not constant and is positively correlated to the moisture content as in this case capillary forces also have to be taken in consideration:

$$K=K(\theta)$$

Where θ is hydric content and K is the hydraulic conductivity. Darcy's law in a non porous medium could be extended as:

$$H=z-\rho(\theta)$$

Where θ is hydric content and ρ is suction load.

Thus in a non saturated medium when hydric content decreases, suction load and piezometric load decrease suggesting a water flux directed from higher moisture content spots to lower moisture content spots due to capillary forces.

HC can be defined as the meter per day of water seeping into the soil under the pull of gravity or under a unit hydraulic gradient (103). The definition points out that it accounts both for soil type and for the forces involved, distinguishing it from the infiltration rate, which do not accounts for these parameters.

2.3.1 HC measurements in BSC. HC measurements in lab conditions were performed with a tension infiltrometer (Decagon Devices, Pullman, WA) which is used for field measurements. It is constituted by two chambers, the upper (or bubble chambers) controls the suction, the lowest contains water that infiltrate the soil at a rate that is determined by the suction selected in the upper chamber. The lowest chamber has a porous sintered steel disk which does not allow leakages in open air. The Infiltrometer is placed on the ground and water starts flowing. The water level in the lowest chamber dropping down can be measured thanks to the graduated glass. The outcoming levels of water measured after equal intervals are elaborated in a spreadsheet.

HC is calculated following the method proposed by Zhang et al. (104) which fits for dry soils. The spreadsheet helps measuring cumulative infiltration vs. time and fitting the results with the function:

$$I=C_1t+C_2t^{½}$$

Where C_1 (ms^{-1}) is related to HC and C_2 ($m\ s^{½}$) is related to soil sorptivity. HC (K) is:

$$K= C_1/A$$

Where C_1 is the slope of the curve of the cumulative infiltration vs. the square root of time and A is a value considering van Genuchten parameters for a given soil type, the suction rate set on the Infiltrometer and radius of the infiltrometer disk which is

2,2cm. *A* value thus accounts for soil texture (12 classes according to USDA nomenclature) and for suction load.

This method was optimized at Arizona State University for laboratory measurements of collected samples of BSC.

Samples were collected in P100 Petri dishes with the crust being in the first few top millimeters. Since any disturbance to the sample could have turned out in surface cracks altering the rheological properties, the measurement of HC was performed directly on the sample contained in the Petri dishes. Measurements were always repeated at least three times to have a significative SD. Samples were always assayed after they were completely dried overnight at 31-33°C in a ventilated oven to maximize the accuracy of the method.

This optimized method turned out to be reliable for HC measurement on BSC in lab conditions as it allows fast measurements using low water quantities and allows to preserve the integrity of the samples which remains always confined.

Hydraulic conductivity was evaluated for samples collected in Chihuauan desert, Mojave desert, Colorado Plateau and Sonoran desert and the values were analyzed to evidence if and how they are affected by EPS (see chapter 4).

2.4 Pigment extraction and quantification.

MAAs and scytonemin are important molecules allowing microorganisms to tolerate high solar irradiation (see paragraph 1.2.2). The synthesis of these pigments can be elicited with UV exposure, representing an adaptation strategy for exposed surfaces colonization. Many cyanobacterial strains secrete scytonemin and MAAS and the increase of these compounds is observable after extraction and spectrophotometric scan.

Scytonemin is extractable in absolute acetone at 4°C in the dark. Grarcia-Pichel and Castenholz (87) proposed a series of trichromatic equations developed by the authors allowing to calculate the corrected absorbance (A*) at 384 nm where scytonemin contributes the maximum absorbance, at 490nm for carotenoids and 663nm for chlorophyll a . To make these calculations all the three absorbances are needed.

The corrected absorbance at 384 nm (scytonemin) is calculated as:

$$A^*_{384} = 1{,}04\ A_{384} - 0{,}79\ A_{663} - 0{,}27 A_{490}$$

where absorbances at the three wavelengths are multiplied by fixed factors. The absorbance at 750nm was subtracted to every measured absorbance to correct for residual scattering. The corrected absorbances for chlorophyll a and carotenoids respectively are:

$$A^*_{663} = 1{,}02\ A_{663} - 0{,}027\ A_{384} - 0{,}01 A_{490}$$

$$A^*_{490} = 1{,}02\ A_{490} - 0{,}08\ A_{384} - 0{,}026\ A_{663}$$

MAAs are extractable with 20% v/v methanol from 1h to 2h at 45°C. Specific content can be determined from the aqueous methanol solution obtained from the extraction with a spectrophotometer measuring the absorbance at the wavelength of MAAs maximum absorbance. The corrected value (A^*_λ) is calculated with the equation (105):

$$A^*_\lambda = A_\lambda - A_{260}\,(1{,}85 - 0{,}005_\lambda)$$

Where A_λ is the measured absorbance at the maximum and λ is the wavelength of the maximum absorbance. This correction is essential to account for water soluble cell matter that also show an absorbance.

Pigment concentration can be expressed as *A* (absorbance or optical density). One *A* corresponds to the value of the absorbance when a compound is dissolved in 1ml solvent and measured with 1-cm-pathlenght cuvette.

Absorbance is normalized on dry weight to calculate specific content.

In alternative specific contents can be expressed using specific extinction coefficients (expressed as liters g^{-1} cm^{-1}) which are 92.60 at 663nm for chlorophyll *a* (106), 112.6 at 384nm for scytonemin (88), 250.0 at 490nm for carotenoids (107) and 17.0 at 312 nm for MAAs (108).

3. Characteristics and role of the exocellular polysaccharides produced by five cyanobacteria isolated from phototrophic biofilms growing on stone monuments

F.Rossi, E. Micheletti, P. Albertano, R. De Philippis

3.1 Introduction

Biofilms growing on lithic surfaces of monuments have been intensively studied over the last decades since they represent a matter of concern for the preservation of cultural heritage due to the severe damages which are caused by their diffusion (63, 109, 110). Biological activity, which has been estimated to be the cause of 20 % to 30% of the deterioration of monuments (111), leads to the staining of the lithic surfaces and to the formation of mineral crusts as well as of the decay form called *patina*, which are the result of chemical and physical interactions of the biofilm with the substrate (64, 113). According to some authors, phototrophs (i.e. cyanobacteria and micro green algae) are the first colonizers of stones (66, 113, 114), being in many cases visible as anti-aesthetic dark pigmentation due to the presence of chlorophyll and carotenoids (70). Furthermore, during the growth the photosynthetic biomass releases carbohydrates, growth factors and other nutrients which can be used as substrates by chemoorganotrophic bacteria that, due to their metabolic activities, may produce corrosive organic acids or oxidize metal cations, causing the decay of the stones and the enhancement of the biodeterioration processes (115).

The synthesis and excretion of Extracellular Polymeric Substances (EPS), mainly produced by cyanobacteria, is a key to the further diffusion of biofilms, but also an enhancer for the deterioration processes of the stones. EPS, which constitute from 50% to 90% of the organic carbon of the biofilm and are primarily composed of polysaccharides (1, 19), represent a framework conferring to the biofilm stability and cohesion, protection against attacks by protozoans and environmental dryness and also a shield against harmful ultraviolet (UV) solar rays due to the possible presence in it of UV screening compounds mainly mycosporine amino acids (MAAs) and scytonemin (42, 116). The presence of this slimy layer is essential for the biofilm,

which in this way is protected and structured, but represents a treat for the preservation of the lithic surfaces of the monuments, which are subjected to mechanical stresses (117, 118) and chemical interactions (64) that can cause changes in their structure and unaesthetic visible marks.

This work was aimed at understanding the possible role of five exopolisaccharide-producing cyanobacterial strains in biodeterioration of lithic faces of the Indian stone monuments and in their contribution in enhancing the resistance of biofilm to stressing environmental conditions. The cyanobacteria were isolated from phototrophic biofilms exposed to intense solar radiation on lithic surfaces of Parasurameswar Temple and of Khandagiri caves, located in the state of Orissa, India.

3.2 Material and methods

3.2.1 Isolation of the strains. Five cyanobacterial strains were isolated from biofilms growing on stone monuments located in the state of Orissa, in the eastern region of India. Biofilm samples were collected from temple of Parasurameswar (20º 20' 6.4" N) and Khandagiri cave complex (85º 44' 48" E). Sampling was carried out by gently scraping with a sterile blade and needle, and the resulting material was stored in screw-cap sterile specimen bottles. Axenic cultures of the organisms were obtained by dilutions and repeated cultivation in agar plates; single cell colonies were repeatedly picked and transferred to fresh medium, yielding several uni-cyanobacterial isolates. The cultures were grown in an orbital incubator (Gallenkamp, Loughborough, UK) with constant stirring at a temperature of 30±1 °C and a continuous illumination of 2.18 W/m^2, provided by cool white fluorescent tubes.

3.2.2 Light microscopy. Cells were observed with a Reichert-Jung Polyvar photomicroscope (Vienna, Austria) using Nomarski differential interference contrast after being stained with Alcian blue solution (in 3% acetic acid) (119).

3.2.3 Identification of the strains. Cultures identification is on the way to be completed

3.2.4 Isolation of the polysaccharides. The polysaccharide released and solubilized in the culture medium (released polysaccharide- RPS) was obtained by centrifuging the cultures. The supernatant was then dialyzed for 24 h against distilled water (changed every 8h) in order to remove medium salts and small particulate. For this purpose, dialysis tubings (12-14000 Da cut off, Medicell International Ltd, London) were used. Polysaccharides were precipitated from the supernatants by adding 97% cold ethanol and desiccated by air-drying.

3.2.5 Determination of anionic density. Aliquots of cultures were dialyzed for 24 h against distilled water before being centrifuged at 3000 x *g* for 15'. Anionic density was evaluated both on pellets and on RPS solubilized in the supernatant, following the method described by Ramus (120) as modified by Bar Or and Shilo (121).

3.2.6 Determination of calcium, magnesium and iron (II) removal. Cyanobacterial cultures, confined in natural cellulose dialysis membranes (12-14000 Da cut off, Medicell International Ltd, London), were dialyzed for 24 h against distilled water (changed every 8h) in order to remove salts present in the culture medium. The dialysis tubings containing the confined biomass were dipped into solutions containing calcium and magnesium or iron metal (10 ppm) solutions for 24h. A control of the adsorption of the confining system devoid of the biomass was made by dipping dialysis membranes containing the same amount of growth medium in 10ppm metal solutions after a 24h dialysis against distilled water. The amount of metal removed by the blank and the biomass was measured by using an Atomic adsorption spectrometer (SpectrAA plus; Varian inc, CA, USA). Specific metal removal (q), expressed as mg metal removed per g of biomass dry weight, was calculated using the following equation (122):

$$q = V(Cb-Cs)/m \ ;$$

where *V* is the sample volume (L), *Cb* and *Cs* are respectively the metal concentration in the blank and the metal concentration (mg/L) in the sample after the

contact with the biomass and *m* is the biomass dry weight (g). The tests were performed in triplicate and the resulting standard deviation is reported in the results.

3.2.7 Determination of the monosaccharidic composition of the RPSs. Aliquots of RPS were hydrolyzed with 2N trifluoroacetic acid at 120°C for 120' and then cooled on ice. Samples were then ultrafiltered with centrifugal filters (Amicon Ultra-4) at 8200 x *g* for 20', dried in a rotary evaporator and then analyzed by using a Dionex ICS-2500 ion exchange chromatograph (Sunnyvale, CA), according to the method reported by Micheletti et al. (123).

3.2.8 Ultraviolet (UV) exposure experiments. Cultures for UV experiments were acclimated in Pyrex flasks under continuous irradiation (2.18 W/m^2 VIS, 0 W/m^2 UV-A, 0 W/m^2 UV-B) produced by cool-white fluorescents bulbs. Aliquots of exponentially growing cultures were put in uncovered petri dishes with BG-11 medium (124) at 30 °C and pretreated for 3 days under three different light conditions: only PAR (2.18 W/m^2); PAR (2.18 W/m^2) and UVA (3.20 W/m^2), with a peak at 360 nm; PAR with UVA and UVB wavelengths (2.18 W/m^2, 1.36 W/m^2 and 2.96 W/m^2, respectively). After the three different pretreatments, the cultures were irradiated at 260 nm for 1 hour with an UV-C germicidal lamp, with an irradiance of 5.13 W/m^2, under constant temperature and low-irradiance conditions (2.18 W/m^2 VIS, 0.14 W/m^2 UV-A, 0.17 W/m^2 UV-B). Test cultures were exposed to UVC, whereas control cultures were covered with pirex lids to block UVC irradiation.

3.2.9 Pigments extraction and quantification Samples (about 50 mg dry weight) of the tested cultures were extracted with 100% acetone overnight and extracts were clarified by centrifugation. Absorbance was measured at 384 for scytonemin, carotenoid pool, and chl *a* content. Correction for residual scatter was done by subtracting the absorbance at 750 nm to every measured absorbance. Scytonemin contents were calculated from the corrected absorbances using the trichromatic equations of Garcia-Pichel and Castenholz (87). For mycosporine-like aminoacids (MAAs) extraction, about 50mg dry weight of sample were extracted with 25% (vol/vol) methanol from 1 to 2h. Specific contents were obtained measuring the

absorbance at the MAAs maximum absorbance of 334nm and correcting the value using the equation reported by Garcia-Pichel and Castenholz (105). Scytonemin and MAAS and contents were expressed as units of absorbance normalized to dry weight ($A_\lambda\ g^{-1}$).

3.2.10 Chlorophyll fluorescence measurements. In order to evaluate the physiological status of the cyanobacterial strains during changing irradiance, photosynthetic activity was determined by measuring the variable chlorophyll (Chla) fluorescence of photosystem 2 (PSII). The effects of ultraviolet radiations UVA, UVA+UVB, and UVC on PS II in terms of electron transport rate (ETR), were estimated using pulse amplitude modulated (PAM) fluorescence. A portable fluorometer Mini-PAM/F (Pulse-Amplitude-Modulation Walz, Germany) was used for measurements. The PAM probe was placed perpendicularly at a distance of about 10 mm from the sample to yield a sufficient signal from the cells. The saturating light pulse was 5300 μmol m^{-2} s^{-1} with 0.8 s duration. Measuring light is about 0.3 μmol m^{-2} s^{-1}, and the actinic light 10 μmol m^{-2} s^{-1}. For quenching analyses, the actinic light was directed to the sample using the same probe.

3.3 Results

3.3.1 Characterization of the isolates. Five cyanobacterial strains have been isolated, three (named temporarily P2a, P2b and P2n) from the biofilm collected on Parasurameswar temple walls and two (named temporarily P3 and P4) from Khandagiri caves. Three strains are unicellular (P2b, P2n and P4) while the others are filamentous. Light microscopical observations after staining with Alcian blue showed a dense blue coloration surrounding the cells of all the five cyanobacteria (figure 9). In the case of strains P2b and P4, the dye is also bound to the material released in the medium, suggesting the presence of anionic compounds both in the layer tightly bound to cells and in the solubilized RPS found in the culture medium.

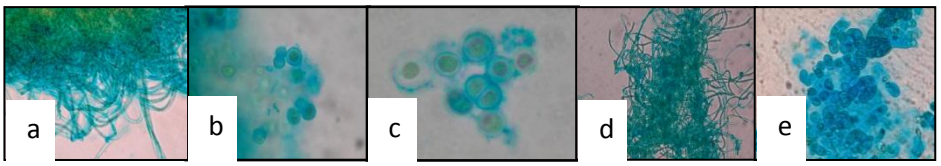


Fig.9. *Light microscopy micrographs of the strain P2a (a), P2b (b), P2n (c), P3 (d) and P4 (e) after staining with Alcian Blue (3% in acetic acid).*

The direct measurement of the anionic charge density on the pellet (i.e. cells + sheath) and in the supernatant (i.e. solubilized RPS) of the cultures of the five strains showed that in strain P3 the negative charges are mostly present on the external cell layer, while in the case of strain P2b and P2n, the negative charges seem to be more concentrated in the released polysaccharides (Table 3). Strains P2a and P2n showed the lowest amount of charges all over its biomass.

Table 3 *Anionic density of the released polysaccharides (RPS) and of the residual biomass (pellet) of the five cyanobacterial strains isolated. The strains were grown under the same specific conditions.*

Strain	***Anionic density[a]***	
	RPS*	***Pellet****
P2a	*0.23±0.08*	*0.12±0.03*
P2b	*0.91±0.16*	*0.1±0.02*
P2n	*0.27±0.09*	*0.1±0.03*
P3	*0.13±0.04*	*0.48±0.12*
P4	*0.24±0.10*	*0.15±0.04*

[a]n=3 ± SD

**=mg of Alcian Blue bound for mg RPS or pellet dry weight*

3.3.2 Identification of the isolates. Strains identification is on the way to be completed.

3.3.3 Monosaccharidic composition of RPSs. Ion exchange chromatographic analyses of the hydrolyzed RPSs showed that the polysaccharides produced by strains P2a, P2n, P3 and P4 are composed by twelve different types of monomers while the RPS produced by strain P2b is composed by eleven types of sugars (Table 4). The aldohexose glucose is present in large quantity, its global amount representing from 20 to 42 % of the moles of sugars in the RPS. The ketohexose fructose was found in all the RPS except in the one produced by strain P2b. The aldopentoses xylose, ribose and arabinose were always found, the former representing from 11 to 24 % of the moles of sugars. The deoxy-sugars rhamnose and fucose were also found, the latter being the sugar present in the highest amount in the RPS produced by strainP2b. The acid sugars, glucuronic and galacturonic acids, and glucosamine were found in the polysaccharides released by all the five strains.

Table 4 *Monosaccharidic composition (expressed as micromoles of monosaccharide per gram of RPS dry weight) of the polysaccharides released in solution (i.e. RPS) by the five cyanobacterial strains. Data shown are mean values from at least three determinations; SD never exceeded 5%.*

Strain	**Monosaccharides**												
	Fuc	*Rha*	*GalN*	*Ara*	*GlucN*	*Gal*	*Glc*	*Man*	*Xyl*	*Fru*	*Rib*	*GalAc*	*GlucAc*
P2a	9.3	5.0	1.3	1.2	0.4	0.7	28.0	n.d.	10.0	4.2	8.26	11.4	0.2
P2b	74.0	4.5	3.5	5.9	3.7	10.0	26.0	n.d.	4.5	n.d	3.0	6.3	20.2
P2n	4.4	6.9	5.9	1.3	14.5	10.9	78.0	n.d.	44.3	1.9	3.2	7.4	8.2
P3	9.7	1.3	0.2	4.0	3.6	13.0	22.0	n.d.	10.0	2.0	2.3	12.0	0.1
P4	7.0	13.0	0.2	30.0	5.0	10.0	30.0	n.d.	30.0	0.9	6.7	12.0	3.1

Fuc, fucose; *Rha*, rhamnose; *GalN*, galactosamine; *Ara*, arabinose; *GlucN*, glucosamine; *Gal*, galactose; *Glc*, glucose; *Man*, mannose; *Xyl*, xylose; *Fru*, fructose; R*ib*, ribose; *GalAc*, galacturonic acid; *GlucAc*, glucuronic acid; *n.d*., not detected.

3.3.4 Calcium, magnesium and iron (II) removal. The five cyanobacterial strains showed good affinity for the divalent ions tested, with different values of the specific removal *q* for each metal (figure 10). Strains P2b and P3 showed the highest specific removal for calcium and magnesium, Ca being the metal removed at the highest *q* value. On the other side, strain P2a showed the lowest *q* values for the three metals in comparison with all the other strains. Except for strain P2n, which showed a similar *q* value for calcium and magnesium (8.13±5.28 and 9.16±1.46 mg/g, respectively), the other strains showed *q* values for Ca two to four times higher than for Mg. Strain P2n showed a high affinity towards Fe (II), showing a *q* value 30 times higher than the ones showed by the other strains and 9 times higher than its *q* values for Ca and Mg.

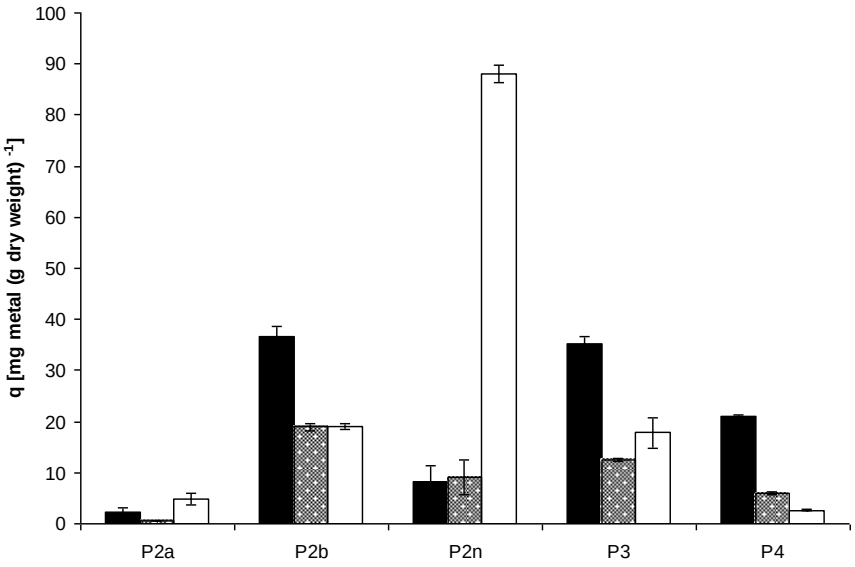


Fig 10. *Specific metal removal q (expressed as mg of metal adsorbed per gram of dry biomass) of the five strains towards Ca^{2+} (black bars), Mg^{2+} (grey bars) and Fe^{2+} ions (white bars). All the values are mean of at least three replicates ±SD*

3.3.5 Protection of photosystem II activity. All the five strains showed complete inhibition of the electrons transfer from photosystem II to photosystem I if exposed to 1h of UVC irradiation (figure 11).

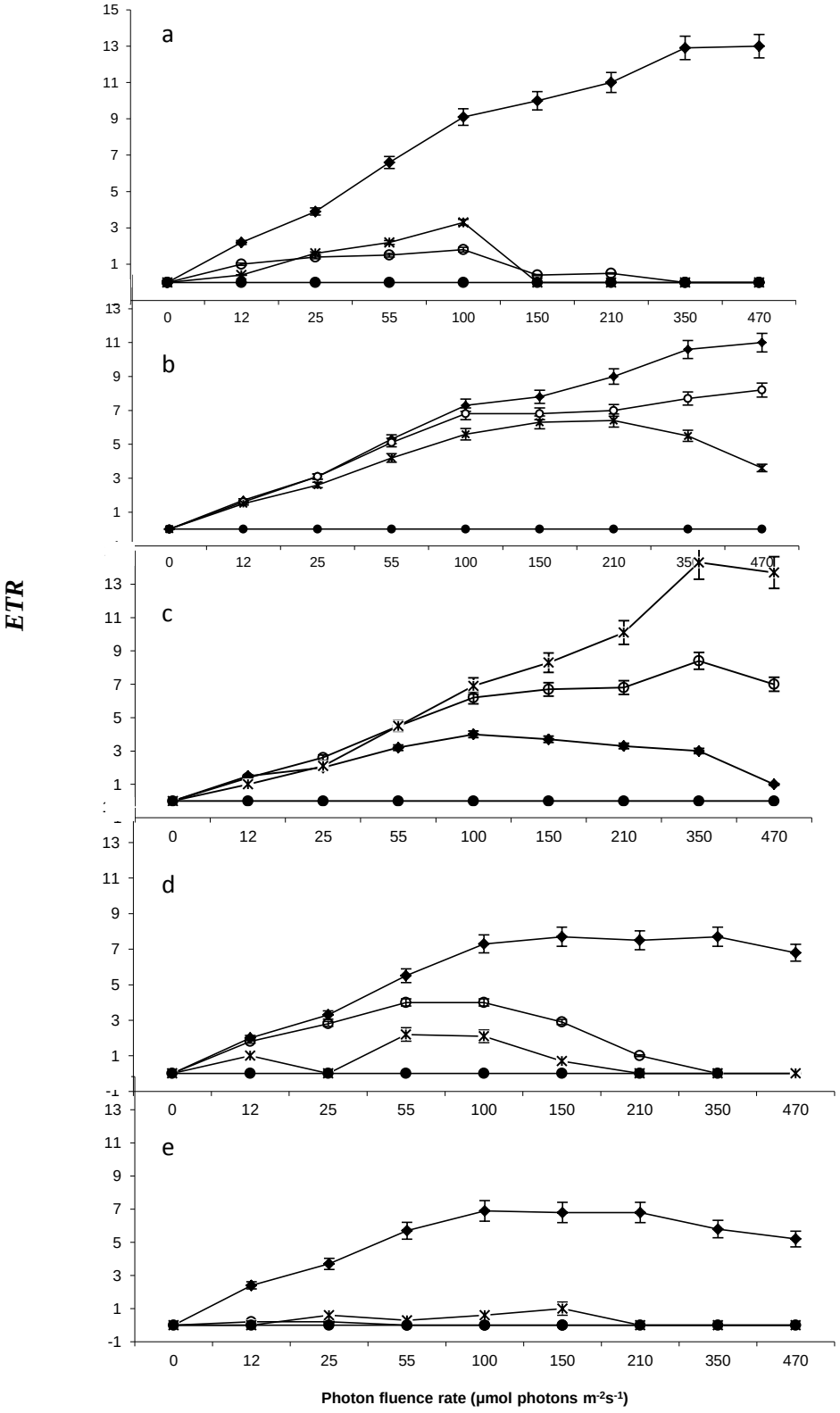


Fig 11. *Electron Transport Rate (ETR)expressed as (µmol e-$m^{-2}s^{-1}$) versus irradiance (µmol photons m^{-2} s^{-1}) of strain P2a (a), P2b (b), P2n (c), P3(d) and P4 (e) in control cultures (not exposed to any UV irradiation) (♦), directly exposed to 1h UVC (●), after 1h of exposure to UVC after three days of irradiance with UVA (○), after 1h of exposure to UVC after three days of irradiance with UVA+UVB (×)*

However, after three days of pretreatment consisting of exposure to UVA or UVB, strains P2a, P2b, P2n and P3 showed to have improved their tolerance to UVC, as they still show partial photosynthetic activity after 1h exposure compared to cultures exposed to UVC without previous pretreatment. In the case of strain P4 no significative improvement in Electron Transport Rate (ETR) came with UVA and UVA+UVB pretreatments. After UV irradiation all the strains except P2n showed a decrease in ETR if compared to control cultures whereas P2n shows an higher photosynthetic capacity with an ETRmax of 14,3 measured after being exposed to UVC after UVA+UVB pretreatment.

P2a control culture reached photoinhibition at PFD values of 350 mmol (photons) m^{-2} s^{-1}, corresponding to an ETR value of 12.9 µmol (electrons) m^{-2} s^{-1}. If adapted to UVA or UVA+UVB the PFD inducing photoinhibition was registered at 100, with an ETRmax value of around 2.1, 3.8 fold less in comparison with the control. Strain P3 control culture reached photoinhibition level at value of PFD of 150, corresponding to a value of electron transfer rate of 7.7. Also in this case exposure to UV radiation brought to a decrease of the photosynthetic activity. Values of PDF exceeding 55, in fact, caused photoinhibition and ETRmax was 1.7 and 2.2 fold less respect to control for UVA and UVA+UVB respectively.

Strain P4 showed the lowest values in terms of ETR. Photoinhibition was reached at PAR 100 with ETR max of 3.9.

For strain P2b UVA exposure did not cause photoinhibition even at the highest actinic light tested, reaching values of ETR of about 8, while in the case of irradiation with UVA+UVB a decrease in electron transfer rate was observed at PFD 210 corresponding to an ETRmax of 6.4.

3.3.6 Extraction of UV screening pigments from the biomasses exposed to UV irradiation. Pigments extraction and quantification revealed that the exposure to UV radiations caused an increment in absorbance at those wavelengths most likely corresponding to sunscreen pigments mycosporine aminoacid-like substances (MAAs) and scytonemin (figure 12). An increase of the corrected absorbance at 384 (contribute of scytonemin) and at 334nm (contribute of MAAs) was observed for strains P2a, P2n and P3 while P2b and P4 showed only an increase of the corrected absorbance at 334nm.

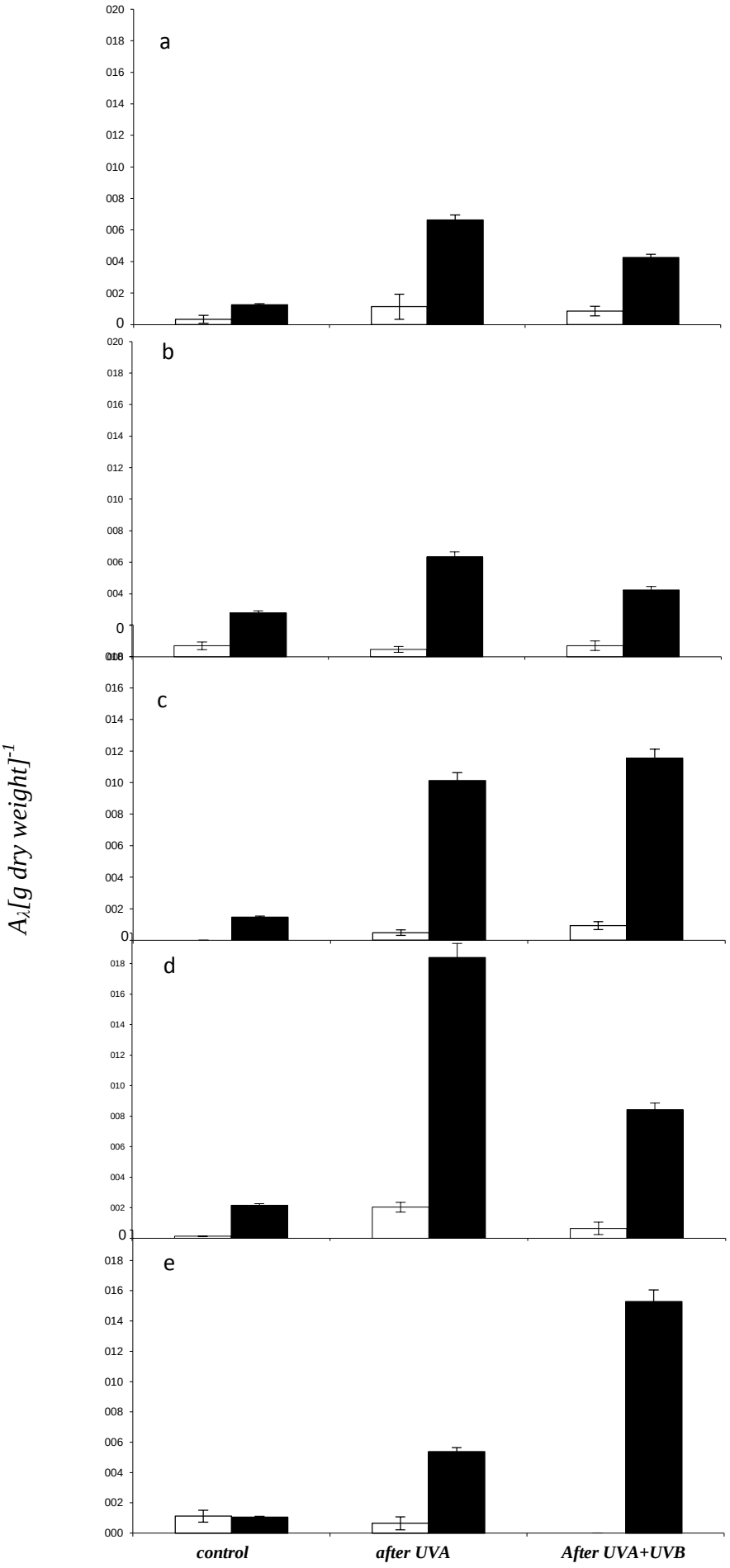

a
b
c
d
e
control
after UVA
After UVA+UVB
$A_\lambda[g\ dry\ weight]^{-1}$

Fig.12 (previous page). *Corrected absorbances normalized to dry weight (Aλ[g dry weight]$^{-1}$) measured at 384nm (where contributes scytonemin)(white bars) and at 334nm (maximum absorbance of MAAs) (black bars)*

After exposure to UVA an increase of the corrected absorbance at 334nm was always registered while an increase of corrected absorbance at 384nm was observed only in the case of strains P2a, P2n and P3.

The double exposure, if compared to the single exposure, brought to a further increase of the corrected absorbance at 334nm in the case of strains P2n and P4, while it brought to a decrease of corrected absorbance in the other cases.

UVA+UVB exposure did not seem to elicit scytonemin synthesis as no significant corrected absorbance increase at 384nm was observed if compared to UVA irradiation whilst a decrease of corrected absorbance was observed in the case of strain P2a, P3 and P4.

3.4 Discussion

The EPS produced by the five strains isolated from the biofilms growing on the walls of Parasurameswar temple and Khandagiri caves resulted composed of at least eleven different sugars. According to the data available on the so far studied cyanobacterial polysaccharides, only 8% of these polymers is characterized by a number of different types of monosaccharides of 10 or more (42, 125). Thus, it seems that a peculiar feature of the EPS produced by the five cyanobacteria studied is their complexity, which could give specific advantages to the microorganisms residing in the biofilms growing on the monuments. Most probably, the presence of a large number of different kind of monosaccharides may represent a big trophic resource for the whole microflora embedded in the matrix of the biofilm when the polymers are degraded. Furthermore, the presence of a large number of different monosaccharides can cause a number of different interactions with the environment due to the specific chemical properties of each sugar. Indeed, the deoxy-sugars rhamnose, which was found ranging from 6 to 9 moles % on the total moles of polymeric sugars in all the EPS studied, and fucose, which was found ranging from 5

to 12 moles %, are known to confer hydrophobic properties to the polysaccharides (126, 127), thus increasing the cohesiveness of the biofilm and its capacity to attach to solid surfaces (1, 128). On the other hand, the rather high anionic density found in the polysaccharidic external layers of the five cyanobacteria studied is capable to confer them a high hydrophilic character. This feature is surely due by the presence of uronic acids, found in all the polysaccharides in the range 8 to 28 moles % of the total EPS moles and identified as glucuronic and galacturonic acids, which are known to contribute either to the anionic nature and to the sticky character of the polysaccharides (100, 129, 130, 131). The simultaneously hydrophobic and hydrophilic nature of the polysaccharides constituting the slimy layer surrounding the cells is capable to facilitate both the adhesion of the biofilms to the stone surfaces of the monuments and the storage of the water occasionally available, thus giving to the producing cyanobacteria and to the microorganisms residing in the biofilms the possibility to cope with the unfavourable growth conditions due to drought periods.

Negatively charged polysaccharides are also known to be capable of strongly binding metal cations, acting as chelators and causing the accumulation in the sheaths of several metals, including Ca, Mg, and Fe (II) (81, 100, 132).

Under laboratory conditions, all the five cyanobacteria showed the capability to absorb calcium, magnesium and iron from water solutions. It was also found that the specific metal uptake *q* for Ca and Mg is in direct correlation with the anionic density measured for the biomass of the five cyanobacteria. This result strongly suggests that the interaction metal/biomass is mainly based on ionic forces developed between the negative charged molecules present in the polysaccharidic external layers and the metal ions. However, this direct correlation was not found in the case of Fe, which was removed at the highest *q* by strain P2n, which showed one of the lowest value of anionic density. This result could be explained considering that the ability to chelate metal ions is related not only to the amount of negatively charged groups present on the EPS, but also to their distribution on the macromolecule and to their accessibility (129, 133).

The stones utilized for the construction of Parasurameswar temple and the rocks where Khandagiri caves were dug are composed mainly of SiO_2 (67.33 %) and Al_2O_3 (18.86%). Calcium, magnesium and iron (II) are present as minerals and represent respectively the 0.5%, 1.02% and 4.01% of the rocks. The affinity showed by the

five cyanobacterial biomass towards Ca, Mg and Fe is likely to provide the microorganisms residing in the biofilm with these metal ions, present both as traces and as oxides that, trapped in the slime, can be utilized both for cell metabolism and for the stabilization of the structure of the biofilm (134). Furthermore, the presence of polysaccharides can enhance mineral dissolution by weakening of metal-oxygen bonds and by binding trace metals thanks to acidic polysaccharides. It is known that no mineral dissolution could be enhanced by polymers mainly composed by neutral sugars (135, 136, 137). The accumulation of iron on the cyanobacterial sheath can represent a reserve of this metal in the case of low iron availability (138) but can also provide the cells with a protection against the harmful effects of a high sun irradiation, in some cases coupled with scytonemin (139, 140, 141, 142).

Four strains out of five showed to be capable of colonizing highly exposed environments by acquiring resistance to intense solar irradiation. Low intensity irradiation exposure was shown to be enough to improve the strains tolerance to further harsher radiations. Results strongly hint at isolates secretion of UV protective pigments.

Extraction procedures with methanol and acetone after UV expositions showed increases of corrected absorbance respectively at 334 nm and 384 nm which are most likely to be related to the synthesis of protective pigments such as mycosporine aminoacid-like substances (MAAs) and scytonemin respectively. Many studies in literature show that the synthesis of scytonemin or MAAs can be induced by UVA and/or UVB irradiations (143, 144). While absorbance increases at 334 nm wavelength (corresponding to MAAs) were observed after UVA only and/or UVA+UVB irradiation, not all the cyanobacterial cultures extracts exhibited a significant increase of absorbance at 384nm wavelength (corresponding to scytonemin) respect to control.

In our case the improvement of photosynthetic activity after the treatment has to be ascribed to the contributes of both pigments with MAAs providing for compensation where scytonemin synthesis was minimal or absent. The MAAs protective action is reported to be explicated both as absorbing compound and as a scavenger of oxygen-species coming from the ionizing effect of high energy irradiation (145).

In the case of strain P4 the pretreatment both with single and double irradiation did not bring to an improvement in UVC tolerance. Despite a prominent increase of

corrected absorbance at 334nm after UVC irradiation after the double irradiation pretreatment, a significant improvement of the culture physiological state compared to the non pretreated culture was not observed.

It is the only case in which corrected absorbance at 384 nm wavelength shows a sharp decrease after UVA pretreatment and it is not detectable after UVA+UVB treatments. It is possible that P4 strain needs longer acclimatation times to UV irradiance than the other four strain tested.

Pretreatments on P2n cells was the only case in which UV-pretreated cells showed an higher photosynthesis rate compared to culture exposed only to VIS wavelength The Highest ETR value was obtained with UVA+UVB pretreatment. Similar results were obtained by Holzinger et al. (146) with the green alga *Zygonema*, collected in Ny-Ålesund, Spitsbergen, Norway and naturally exposed to environmental stressing UV radiation. Strain P2n appeared to possess adaptation plasticity as the corrected absorbances related to scytonemin and MAAs secretion show to increase passing from single to double irradiation pretreatment after which, notwithstanding the 1h UVC irradiation, the strain shows the highest level of photosynthetic activity.

In conclusion, the results strongly suggest the strains adaptability to constrained environment such as exposed lithoid surfaces under high solar irradiance.

The polysaccharidic slime released by the five strains showed chemical variability and complexity indicating the given chance to enstablish both hydrophobic and hydrophilic bonds. This assures the biofilm a good versatility in terms of attachment and stability against erosion. The biomasses affinity with bivalent cations suggest their capacity to store them collecting trace metals and/or contributing to weaken substrate minerals, enhancing substrate decay.

4. Role of the released exopolysaccharidic material in the hydraulic conductivity and composition of the microbial community of biological soil crusts from four North American deserts

F.Rossi, H. Beraldi, F. Garcia-Pichel , R. De Philippis

4.1 Introduction

Water movement in dry lands is a critical process for the formation and the maintenance of this peculiar landscapes. As a consequence of the water retaining capability of dry soils, nutrient bare are alternated to substrate-rich patches that are characterized by large differences in their biological activity (147, 148). The patchy distribution of both moisture and trophic resources, characteristic of arid and semiarid environments, reflects the distribution between run-offs and sinking areas, respectively characterized by low and high water infiltration rates (149). Water infiltration depends both on the abiotic characteristics of the soil (e.g. crackings, microtopography and sealing) and on its physical properties, such as particle size and organic matter distribution (97, 150).

Biological Soil Crusts (BSC), widely distributed through these ecosystems, have been studied for decades for their implication in hydrological cycles. However, although a number of studies have suggested a role of BSCs in modifying soil parameters like the degree of particle aggregation and soil porosity (91, 151), unambiguous results regarding how these factors are affected by their presence still lack.

Whilst in some cases crusting of soil surface has been connected to a decrease in water infiltration rate (152, 153, 154, 155) and to an increase in run-offs yields (156), other authors observed an increase of water infiltration in BSC covered soils in comparison with bare soils (93, 98, 157). For instance, studies carried out in different sites in Australia led to opposite results. In sandy-loam soils, a reduction of infiltration in microbiota-covered soils in comparison with bare soil was observed (158), while in degraded massive red earth soils the crusts seemed to work as enhancers of water penetration (98).

These conflicting results point out the complexity of this process, most probably due to the concurrence of many factors, including crust characteristics, their moisture status, the texture of the soil and intensity of the rainfall events which soils are subjected to.

Extracellular Polymeric Substances (EPS), produced mainly by cyanobacteria and green algae (92), have been suggested to be capable of influencing water movement in BSC covered soils. It has been reported that EPS are able to reduce infiltration due to their capacity to clog soil pores in late stages crusts (96, 159, 160), slowering the water flow especially in sandy soils. On the other hand, Mazor et al. (161) and Fischer et al. (162) also pointed out the importance of the hydrophobicity of polysaccharides, mainly due to the chemical nature and the abundance of the exudates, in the early stages of crust development. Nevertheless, other authors reported that polysaccharides can positively affect soil porosity in more than one way. Indeed, it has been reported that the presence of cyanobacterial polymeric exudates in microbiotic covered soils caused an increase in the number of micropores, which are known to increase water infiltration (163, 164), and a modification in the geometry of the pores (165). The production of a sticky matrix trapping airborne particles was also reported to lead to the formation of sand and clay layers which are absent on soils not covered by crusts. Silt and clay particles can increase the sorptivity of the soil helping water infiltration, though they might decrease soil porosity (91).

In order to define the actual role of the polysaccharidic exudates in modifying the hydrological processes in microbiotic crusts, this study was aimed at evaluating the relationships between the presence of exopolysaccharides and the water diffusion in crust-covered soils from four north American deserts, Sonoran, Mojave, Chihuauan and Colorado Plateau, representing extreme arid environments characterized by low medium annual precipitations and by different characteristics of the soils. In particular, the correlations between water movement through the soil and factors such as soil texture and the amount of phototrophic microorganisms were investigated. A preliminary experimental activity aimed at developing a non destructive EPS-extraction method was carried out in order be able to measure the variations in water seeping rates when part of the matrix has been removed.

4.2 Materials and Methods

4.2.1 Sampling. Samples were collected from five sites that will be referred to as North from Soda Lake (NSD) (35 deg 15' 06.6" N, 115 deg 58' 38" W) and Hayden-Globe Road (HGR)(35 deg 02' 10.8" N, 115 deg 34' 31" W), both part of Mojave Desert; Sunday Churt Site (SCS)(38 deg 38' 01.3" N, 109 deg 38' 20.2" W), found in Colorado Plateau; Yuma/Dateland Site (YDS) (32 deg 45' 6.4" N, 113 deg 39' 4.1" W) found in Sonoran Desert and Jornada Range Road (JRR)(32 deg 31' 58.9" N, 106 deg 43' 41.5" W), found in Chihuauan Desert. Four samples were taken from YDS (marked 704, 707, 708 and 710); two samples (marked 1088 and 1089) were taken from SCS; two samples (marked 905 and 883) were taken from HGR and one sample (marked 789) was collected from JRR. Samples were collected carefully displacing them in Petri dishes containing soft paper to avoid cracking and to obtain the collection of the first centimeters of topsoil. Samples were covered with plastic covers and sealed with tape. Before all the experiments samples were oven-dried at 31-33°C overnight to lose the moisture and then stored in the dark.

4.2.2 16S rDNA V4 Pyrosequencing. DNA was extracted from BSC in these sites using the Ultra Clean Soil DNA Extraction Kit (standard or Mega, MoBioLabortories, Inc, Carlsbad, CA). Barcoded bacterial primers 519f and 907r were used according to Schwieger and Tebbe (166) to amplify the V4 variable region of the 16S rRNA gene (supplied by H. Zhang, ASU Biodesign Institute). Amplicon pyrosequencing was performed by the University of Arizona Genetics Core (1657 E Helen St, Tucson, AZ). The pyrosequences were parsed by sample according to the individual barcodes. For data analysis, we used mother pyrosequencing analysis software described by Pat Schloss in the mothur user manual for sequence preparation, and the Ribosomal Database Project (RDP), Release 10 (167), pyrosequencing alignment tool to assign taxonomy. RDP Library Compare (RDP Naive Bayesian rRNA Classifier Version 2.2, March 2010, RDP training set 6 based on nomenclatural taxonomy and Bergey's Manual) was used to estimate the percentage of sequences from each desert sample assigned to the major taxonomic groups (phyla) and to compare them across all U.S. deserts.

4.2.3 Soil texture determination. For each EDTA-treated and non-treated soil samples, three to four 1 cm^3 sub-samples were taken for grain size analysis. Soil sub-samples were disaggregated by hand until no aggregates were seen, then heated at 70° C for two days to remove environmental moisture, and weighed. They were further incinerated in a muffle furnace at 500°C for 6 hours, to remove any organic matter, and weighed again. Weigh differences were used to estimate the total content of organic matter (168). The samples were then sealed in sterile 1.5 mL eppendorf tubes until measurements were done. Grain size measurements were made in triplicates for each sub-sample, using a Malvern Mastersizer 2000 grain size analyzer (Malvern Instruments, Ltd., Malvern, UK) at the Environmental Studies Laboratory at the University of Arizona. Cut off diameters were 2 µm for clay, 2-53 µm for silt, and 53-2000 µm for sand. Each sub-sample was sonicated for 2' before each analysis.

4.2.4 Chlorophyll a extraction. Sediments were extracted in 80% acetone, cells and sediments were pulled down by centrifugation at 2600 x g and absorbance of the extracts was read at 663 nm using a Cary 50 Spectrophotometer (Varian, USA). Absorbance at 750 nm was subtracted to correct for eventual residual scattering. Pigment quantification was evaluated using the specific extinction coefficient of McKinney (169).

4.2.5 Carbohydrate content determination. For total and ethanol-extractable carbohydrates content determination, sediments were gently omogenized and treated with a 0,1M EDTA solution following the method described by Underwood et al (54). High molecular weight polysaccharides were precipitated treating with 70% (v/v) ethanol (final concentration) allowing them to precipitate for 8h at 4°C in the dark. The supernatant is discarded and precipitated sugars re-suspended in deionized water. Carbohydrate content in solution was estimated using Phenol-sulfuric acid assay (101).

4.2.6 Hydraulic conductivity measurements. Hydraulic conductivity accounts for the rate of water movement through soil porous system and accounts for soil's ability to transmit water when submitted to an hydraulical gradient. Hydraulic conductivity was measured on dried samples allocated in Petri dishes using a Mini Disk tension Infiltrometer (Decagon services,Inc., Pullman, WA) with a suction range from 0,5 to 6 cm and a radius of 2,2cm. Hydraulic conductivity was calculated using the method

proposed by Zhang (104) for dry soils and using van Genuchten parameters related to soil texture obtained from Carsel and Parrish (170)**.** For very sample hydraulic conductivity was measured three times drying samples before every measurement at 31-33°C for 12h. Standard deviations were calculated and reported .

***4.2.7 Polysaccharidic matrix extraction*.** Every sample was placed in a plastic Petri dish and 1mm diameter pores were done through the dish basement, coupled by two, at the edge of the basement (figure 13). For this purpose a metal tip pre-entively made hot on a Bunsen was used. Every sample-containing dish was leaned on a second plastic dish and the connection between the two dishes is sealed with silicone in order to obtain a two chamber-structure in which the only internal continuity is given by the performed pores connecting the two chambers. The sample, allocated in the upper chamber is treated with 20ml 0,1M EDTA solution, the surface is covered in order to avoid water evaporation and the two chambers are left for 3h in the dark to allow EDTA to act as a chelator on the sample. EDTA solution acts collecting and removing calcium ions thus destabilizing matrix structure.

Afterwards, a 1mm diameter hole was practiced on the side of the lowest chamber still using an hot metal tip and a Pasteur pipette connected to a void pump was inserted. The pump operates creating a depression ranging from -400 to -600 MPa. Following the suction EDTA solution is forced to flow from the upper to the lowest chamber passing through the sample and moving the slime which leaves the sediments, pass through the holes and collect inside the lower dish before being inspired. A final wash is performed in order to remove residual EDTA and colloids still present after the suction. For these purpose while the void pump is still on, the sample is overflowed with tap water which becomes finally completely removed by the suction of the pump. This latter process on the sample is performed two times and afterwards it is put at 31-33°C overnight to remove completely the moisture.

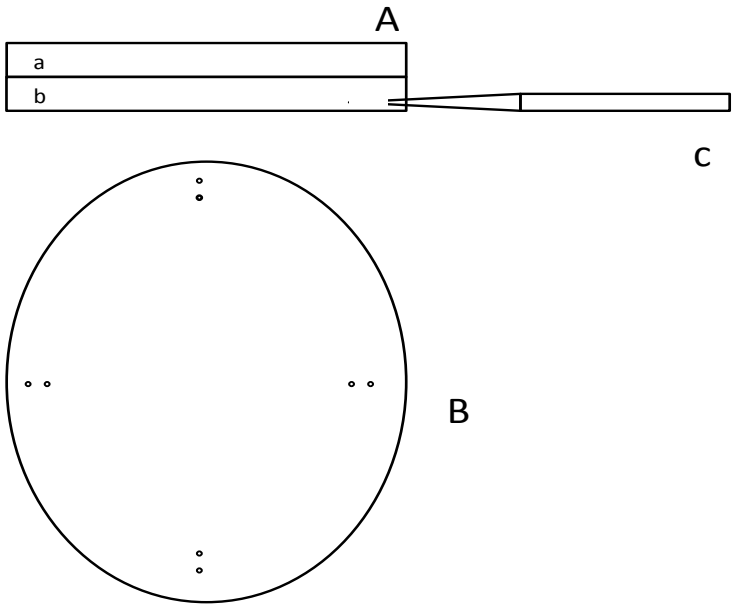


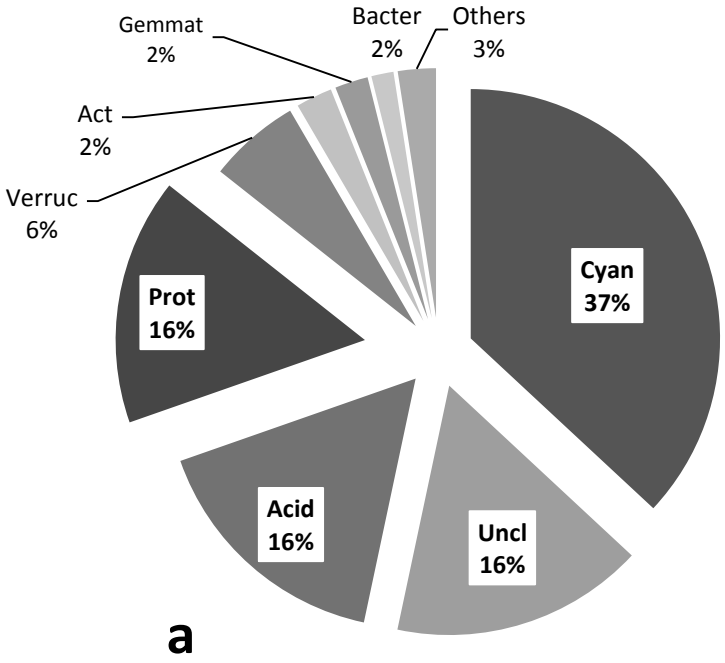
Fig. 13. *Schematic representation of the structure used for the extraction process. A, side view of the two petri dishes showing the upper (a) and the lower (b) dishes with the Pasteur pipette (c) inserted; B upper view of the upper dish showing the holes connecting to the lowest dish.*

4.3 Results

4.3.1 Grain size analysis and composition of the microbial community of the BSCs.

From the grain size analysis, three classes of textures have been identified (Table 1). The soil samples resulted mainly composed of silt and sand, with clay fractions ranging between 0.21% to 3.43%. YDS samples, JJR 789 and HGR 883 resulted to be composed by similar percent ratios of sand and silt, while in SCS samples and HGR 905 sand resulted predominant, reaching the 83% in the case of HGR 905 and the 77-79% in the case of SCS 1088 and 1089. Silt fraction is way lower than in the other samples (16,43% in the case of HGR 905 and 21,76% in the case of SCS 1088 and 1089).

16S DNA pyrosequencing analyses of the BSCs under study revealed a variegated microbial community in terms of phyla, mostly represented by cyanobacteria, proteobacteria and acidobacteria, though the presence of a smaller portion of verrucomicrobia and actinobacteria can also be observed (Figure 14). In most of the sites (Sonoran, Chihuauan and Colorado Plateau sites), cyanobacteria turned out to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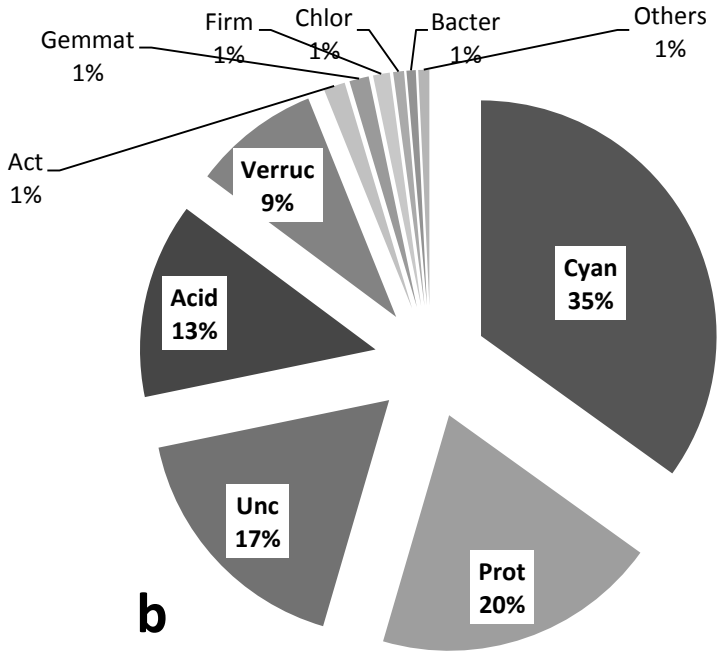
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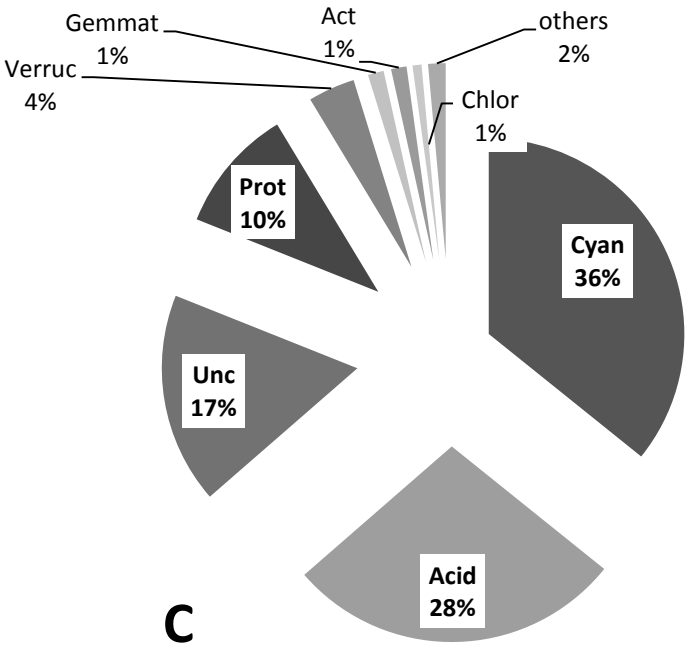
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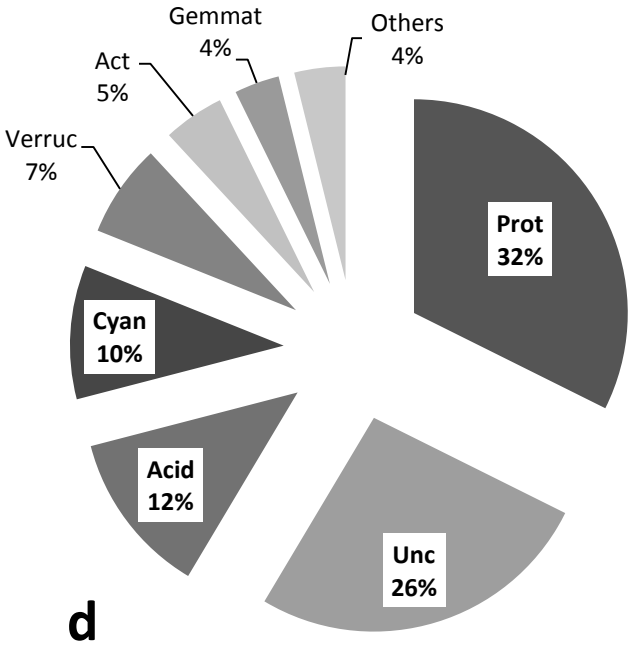
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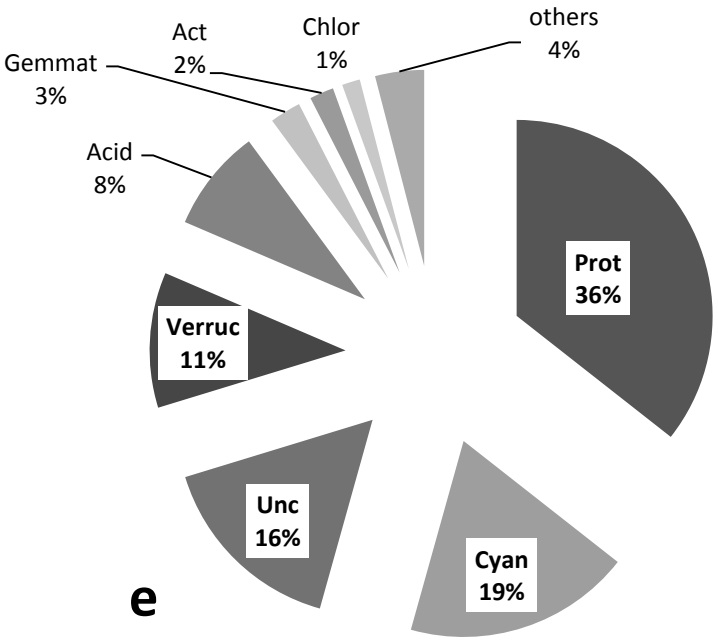

Fig. 14. *Pie charts showing phyla abundances in investigated sites: Colorado Plateau SCS (a), Sonoran desert YDS (b), Chihuauan desert JRR (c), Mojave desert NSD (d) and Mojave desert HGR (e). Abundances are expressed as percent over the total microbial community. Abbreviations:* Prot, *Proteobacteria;* Verruc, *Verrucomicrobia;* Cyan, *Cyanobacteria;* Chlor, *Chloroflexi;* Act, *Actinobacteria;* Gemmat, *Gemmatimonadetes;* Acid, *Acidobacteria;* Unc, *Unclassified bacteria.*

be the relatively most abundant taxonomic group present and they showed to be present in similar percentages (35-37% abundance) while proteobacteria and acidobacteria showed a smaller and also similar abundance (13% to 20%) with the exception of JRR 789 site in which acidobacteria represents the 28% and proteobacteria only 10%. Mojave desert sites showed peculiar abundance distribution compared to the rest of the sampling sites, as they showed α-proteobacteria (32-36%) as the most represented phylum, while cyanobacteria were less represented, reaching only 10% in the case of HGR site and 19% in the case of NSD site.

4.3.2 Hydraulic conductivity and exopolysaccharidic content of BSCs.

For every sample, hydraulic conductivity was evaluated three times, always oven-drying the samples at 31-33°C overnight before the measurements. The hydraulic

conductivity showed to be much different within the samples tested (Table 5), pointing out a significant influence of the soil texture on the capability of water to penetrate the soils.

Table 5 *Hydraulic conductivity for the nine crust samples. Data shown are the result of at least three measurements. Texture classes were assigned according to USDA nomenclature. Results are expressed as(n±SD)10^{-4} cm s^{-1}*

Sample	***Texture***	***Hydraulic conductivity (cm s^{-1})***
YDS 704	*Sandy loam*	*(4,55 ± 1,4) 10^{-4}*
YDS 707	*Silt loam*	*(0,62±0,2) 10^{-4}*
YDS 708	*Silt loam*	*(0,4±0,1) 10^{-4}*
YDS 710	*Silt loam*	*(1,58± 0,3)10^{-4}*
JRR 789	*Sandy loam*	*(0,32±0,12) 10^{4}*
HGR 883	*Sandy loam*	*(6,4± 1,39) 10^{-4}*
HGR 905	*Loamy sand*	*(18,4± 3,3) 10^{-4}*
SCS 1088	*Loamy sand*	*(8,3±2,7) 10^{-4}*
SCS 1089	*Loamy sand*	*(13,9± 6,5) 10^{-4}*

Hydraulic conductivity showed to increase (R^2=0,847) along with the increase of the sand content of soils (figure 15a), whereas an increase of silt percent appeared to be related to a decrease in water movement (R^2=0,855) (figure 15b). Although clay percent was found to be relatively low for all the samples, nonetheless the increase in the amount of clay caused a decrease in the hydraulic conductivity of the soils (R^2=0,647) (figure 15c).

No significant correlation was observed between chlorophyll a content , which represents an index of the phototrophic biomass present in the soils, and their hydraulic conductivity (figure 16).

Carbohydrate content of BSCs was determined both as total and ethanol-precipitable carbohydrates, which represent sugars with molecular weight higher than 100kD (100, 102). The percentage of high molecular weight carbohydrates on the total sugar content ranged from 26 to 35,5% in the samples YDS 704, 710 and in JRR 789,

while they represented a value of 44,4% in HGR 883 and 905 samples.

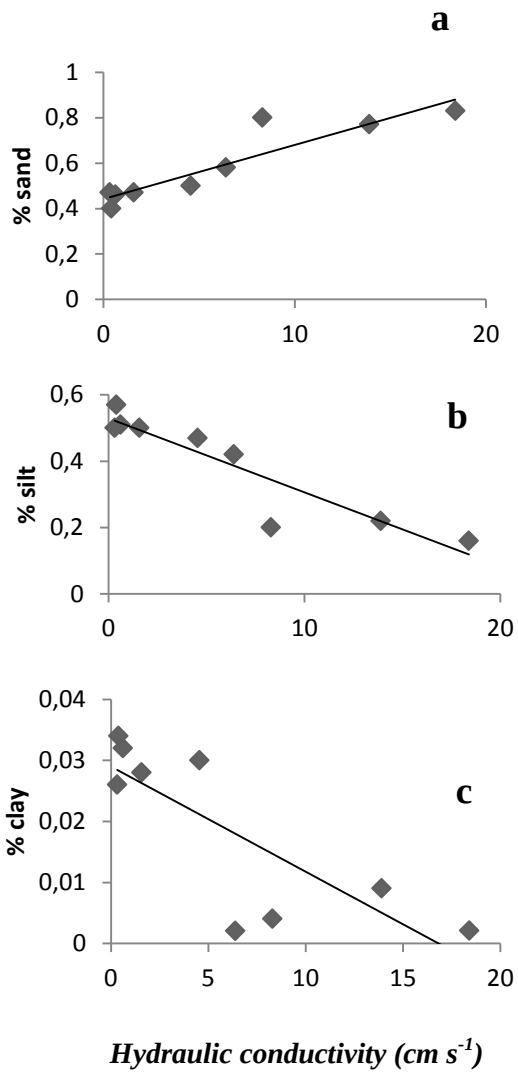

Fig. 15. *Percents of soil size fractions(expressed as n 10^{-3} %) plotted against hydraulic conductivity (expressed as cm s^{-1}) . (a)Hydraulic conductivity vs % sand, (b) hydraulic conductivity vs % silt, (c) hydraulic conductivity vs % clay.*

The percent increased in SCS 1088 and 1089 samples, where high molecular weight sugars represented the 63%.

A significant correlation (R^2=0,676) between chlorophyll *a* and carbohydrate content of the nine BSC samples analysed was found (figure 17a), especially with the content of high molecular weight polymers, even if a rather significant correlation (R^2=0,543) was also observed with the total carbohydrate content. The hydraulic conductivity was found to be negatively affected (R^2=0,532) by the amount of total carbohydrates present in the BSC samples studied (figure 17b). Indeed, the higher the carbohydrate content the lower hydraulic conductivity was, pointing out a slower water flow in the presence of higher amounts of sugars.

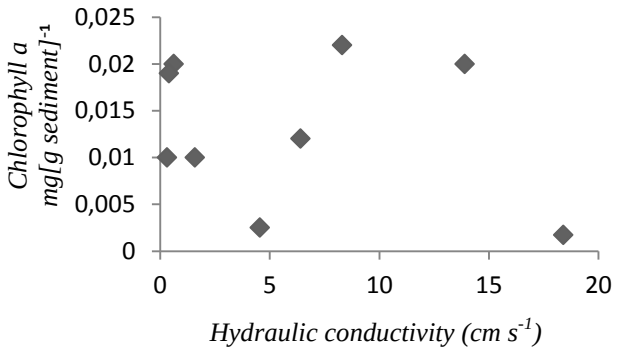

Fig. 16. *Chlorophyll a contents (expressed as mg[g sediment] $^{-1}$) plotted against hydraulic conductivity (expressed as cm s^{-1}).*

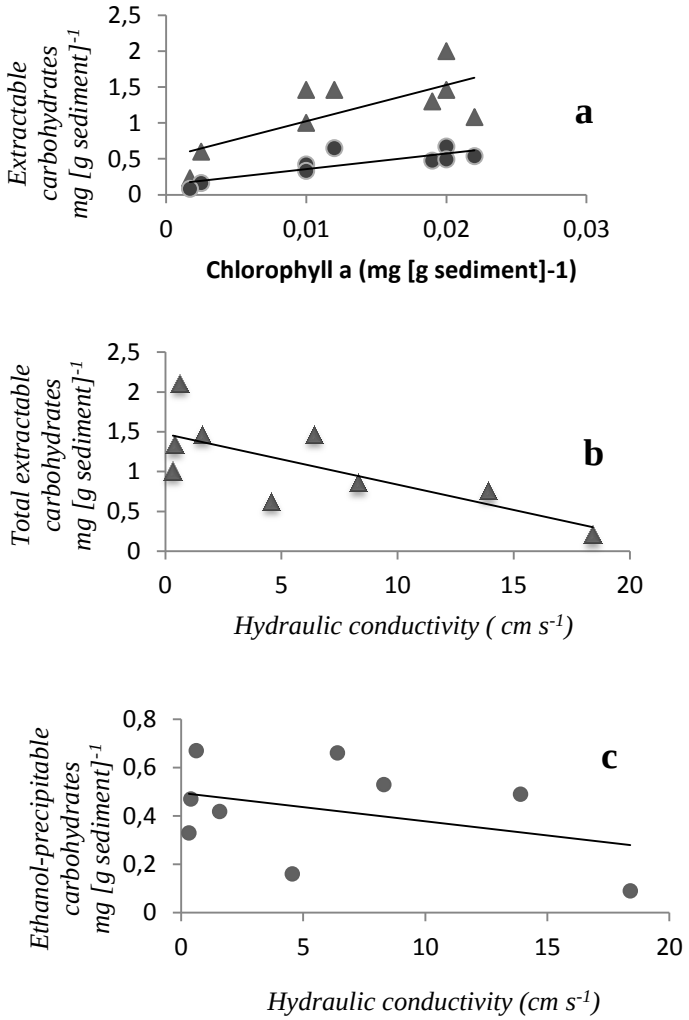

Fig. 17 *Correlation graphs between (a) chlorophyll a content (expressed as mg [g sediment]$^{-1}$) and total (▲) and ethanol- precipitable (●) carbohydrate contents (both expressed as mg [g sediment]$^{-1}$), (b) between total carbohydrate contents and hydraulic conductivity (expressed as cm s^{-1}) and (c) between ethanol-precipitable carbohydrate contents and hydraulic conductivity.*

However, a very poor correlation (R^2=0,1412) between the content of high molecular weight carbohydrates and the hydraulic conductivity was found (figure 17c).

4.3.3 Extraction of the polysaccharidic matrix from BSCs.

Five BSCs, namely YDS 704, YDS 710, JRR 789, SCS 1089 and HGR 905, were treated for extracting the extracellular polysaccharidic matrix by using the method expressly developed for maintaining the integrity of the crusts. After the treatment, a first visible effect of the extraction was a change in the color distribution of dried samples. Indeed, while in the untreated BSCs a light brown colour was homogeneously distributed all along the crusts (figures 18a and 18c), an evident reduction in albedo was observed after the extraction procedure (Figures 18b and 18d), the crusts showing an unevenly dispersed dark brown colour.

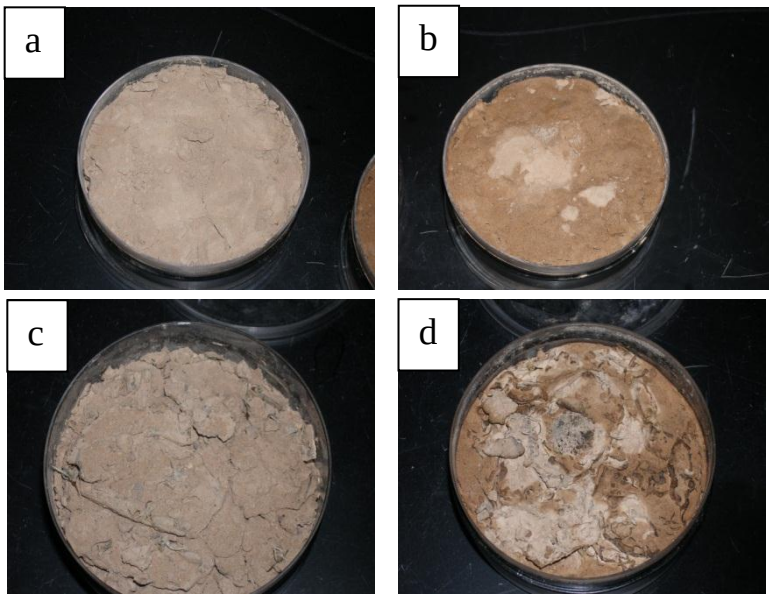

Fig. 17 *Not-treated (a,c) and EDTA-treated samples (b,d).*

The extraction resulted effective for all the five samples, reducing the carbohydrate content per gram of soil in five cases out of five (table 6). Indeed, the content of both total sugars and high molecular weight carbohydrates decreased after the extraction showing that at least a fraction the polysaccharidic matrix was removed. The efficiency in the removal of the matrix from the BSCs was different from sample to

sample, the lowest efficiency having been shown by the silt loam sample YDS 710, which maintained about 90% of its total carbohydrate content after the extraction. However, despite the differences in the grain size distribution of the samples, in all the cases a significant decrease in hydraulic conductivity was observed after the treatment (table 6).

Table 6 *Variations in the HC (expressed as cm s^{-1}) and sugar contents (expressed as mg [g sediment1^{-1}) for the five samples subjected to treatment. Abbreviations: HCb, hydraulic conductivity before treatment; HCa hydraulic conductivity after the treatment; TCb, Total carbohydrate content before the treatment ; TCa, total carbohydrate content after the treatment; EPCb, ethanol extractable carbohydrate content before the treatment; EPCa, ethanol precipitable carbohydrate content after the treatment; - , under the limit of detection of the tensiometer; • At the limit of detection of the tensiometer*

Sample	***HCb***	***HCa***	***TCb***	***TCa***	***EPCb***	***EPCa***
YDS 704	*(4,55 ± 1,4) 10^{-4}*	*(1,0) 10^{-4}*	*0,6*	*0,025*	*0,160*	*-*
YDS 710	*(1,58± 0,3) 10^{-4}*	*(0,91±0,2) 10^{-4}*	*1,46*	*1,31*	*0,418*	*0,323*
JJR 789	*(0,32±0,12) 10^{-4}*	*(0,05)10^{-4} •*	*0,998*	*0,532*	*0,33*	*0,30*
HGR 905	*(18,4± 3,3) 10^{-4}*	*(6,78) 10^{-4}*	*0,232*	*-*	*0,08*	*-*
SCS 1089	*(13,9± 6,5) 10^{-4}*	*(4,8±2,8) 10^{-4}*	*0,751*	*0,27*	*0,49*	*0,16*

4.4 Discussion

The role of biological soil crusts in hydrological cycles in drylands is still controversial and the data reported in literature point out the complexity of defining a precise role for BSCs as many factors, both biotic and abiotic, concur in affecting water penetration and distribution in desert soils. However, the hydraulic conductivities measured on the soil samples studied in this research strongly support the statement that soil texture is the most important factor controlling water infiltration. Indeed, the abundance in sand appeared to be positively correlated with an easier water movement while the amount of silt and clay were negatively correlated with hydraulic conductivity, indicating their opposition to water diffusion, as it was previously reported by Brady and Weil (171), who showed that silt and clay are capable to reduce soil porosity, even if in few cases it was shown that they may enhance soil absorptivity (91).

The amount of phototrophic microflora per gram of dry soil, indirectly quantified through the determination of chlorophyll *a* in the soil, does not seem to significantly affect water movement as no significative correlation with hydraulic conductivity was observed. Anyway, the presence of phototrophic microorganisms appeared to be in positive correlation with the carbohydrate content of the nine soils analyzed and this can be observed both for total carbohydrates (R^2=0.543) and even more (R^2=0.676) for high molecular weight carbohydrates, namely exopolysaccharides. These data strongly support the key role played by cyanobacteria and green algae living in the crusts in synthesizing the polysaccharidic material constituting the matrix connecting cells and soil particles in BSCs.

The significant presence of phototrophic microorganisms, mainly cyanobacteria, was also confirmed by the 16S DNA pyrosequencing analyses of the procariotic community. Comparison with previous data on the microbial communities in these areas shows aligned results with previous reports on community composition by Nagy et al in Sonoran desert (172) and by Garcia-Pichel et al. in the Colorado Plateau (173) who described similar community compositions.

In three deserts out of four, cyanobacteria resulted the most abundant phylum. This is coherent with the fact that these environments impose several constraints and cyanobacteria are known to have developed adaptation strategies allowing to thrive in harsh conditions (174). Nonetheless in Mojave desert samples a minor amount of cyanobacteria was observed while α-proteobacteria resulted dominant. Dong et al also reported in a previous article a predominance of α-proteobacteria over cyanobacteria in Mojave desert microbial communities. The same authors pointed out the capacity of α-proteobacteria to bear with very scarce annual rainfall amount and their having been reported by some authors as putative aerobic anoxygenic photosynthetizers (175, 176), thus being ecologically competitive. In our case a relatively minor cyanobacterial proliferation most probably facilitated their major abundance. Their considerable presence in all the soils tested suggest their ecological importance.

The hydraulic conductivity significantly decreased with the increase in the amount of total carbohydrates per gram of dry soil, suggesting that a larger amount of saccharidic material is capable to reduce the water flow in the soil. This phenomenon is most probably related with the tendency of polysaccharides to swell after retaining

the moisture, thus growing in volume and obstructing the pores with a consequent increasing difficulty for the water to flow through the spaces, as it was previously suggested (96, 159, 160). Indeed, some authors reported that the presence of polysaccharides in the soil causes an increase in water run-off due to the swelling of these polymers in the presence of water as EPS are known to be capable of holding up to five times their weight in water at -1,5 MPa and up to 10 times of its weight in water at -0,5 MPa (162, 165).

In addition, we also tested the correlation between hydraulic conductivity and the amount of ethanol-precipitable carbohydrates, i.e. polymers with a MW larger than 100kD. Interestingly, in this case the correlation with hydraulic conductivity was very poor, even if slightly negative, suggesting a significant role of oligosaccharides in conditioning water movement.

Despite the negative correlation observed, the removal of part of the exopolysaccharidic matrix from the BSC by using the non destructive EPS-extraction method developed in this work caused a reduction of the hydraulic conductivity in all the samples tested, pointing out a higher difficulty for water to penetrate the soils after the removal treatment, instead of showing an increase in infiltration rates. Our data, clearly pointing out that the presence of the polysaccharidic material in the soil contributes to increase the amount of water that can be absorbed (i.e. hydraulic conductivity), might be explained considering not only the hygroscopic nature of polysaccharides and its physic encumbrance, but also looking at a possible structuring role played by these polymers. .

Since hydraulic conductivity depends on the characteristics of the solid matrix of the soils, including shape, number and size distribution of the pores (177), a possible change in these factors due to the extraction of the polysaccharides must be taken into consideration. Indeed, recently it was reported role of cyanobacterial exopolysaccharidic exudates in increasing the number of micropores in biological soil crusts, inducing the formation of pores with a diameter between 0.04 and 50 μm and affecting their geometry (165).

Thus, it can be hypothesized that the extraction of EPS has induced a significant change in micropore number and/or shape, a phenomenon that has been correlated to a decrease in the hydraulic conductivity (91).

Moreover, it is worth mentioning that polysaccharides can induce the formation of surface crackings (96, 178) thus making easier water penetration into the soil; consequently, the removal of polysaccharides might have caused an increase in soil compactness and a collapse of the spatial organization of the pore system. This hypothesis seems to be supported by the significant changes observed in water transmittance and in albedo, which visually suggests a change in the soil surface structure.

From the data reported in this study, it is possible to conclude that the main factor controlling water conductivity in the BSCs studied is represented by soil texture but also the role played by exopolysaccharides is significant in controlling water distribution in extreme environments. On one side, exopolysaccharides provide for moisture captation and storage due to their hygroscopicity, on the other hand, they act structuring the crusts, most likely affecting pore system structure. Thus, in sandy soils the ecological significance of the water retention provided by cyanobacteria exudates is crucial for crust organisms living in arid ecosystems where fast evaporation takes place; on the other side, the structuring of the pore system and crackings formation due to the presence of EPSs is crucial for water penetration in fine-textured soils, which otherwise would be rather impermeable (96).

5. Other studies

5.1 Study on the relationships between microbial-derived exopolysaccharides and water in artificial Biological Soil Crusts from Mongolian desert.

Another study started off during the last months of this PhD comes from a collaboration with the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, consisting in investigations on artificial Biological Soil Crusts in Mongolian desert. The main aim of the study is to assess whether relations with water (e.g. hydraulic conductivity and water captation) are affected by microflora composition and total and high molecular weight, finally evaluating the ecological weight of artificial BSC on water distribution in arid environments. This research could give, for the first time, the possibility to compare the results obtained in the studies carried out on the role of the polysaccharidc matrix in natural BSC with those obtained studying artificial BSC and to point out possible common features.

Analysis concerned the BSC present in desert sites inoculated in various years spanning from 2002 (first inoculated sites) (40. 36619 deg N, 109.84486 E) to 2004 (40.36714 deg N, 109.84479 E), 2005 (40.36643 deg N, 109.85014 deg E) and 2007(40.39231 deg N, 109.81541 deg E); the measurements were carried out both under field and laboratory conditions.

For every sampling site three spots were chosen for field experimentations and samplings that were always carried out within 40 x 40 centimeters squares.

5.1.1 HC measurements. Hydraulic conductivity (HC) was measured in field conditions at the corners and in the center of the squares using a tension infiltrometer (see paragraph 2.3.1). Measurements were performed both on the crust covered soil and on the underlying soil after the removal of the crust to account for the contribute of the bare soil in water penetration. The same suction rate was always applied on the instrument in all the measurements as examined soils presented similar texture and required similar suction load. Final values of HC were not calculated as proper soil texture classes have not been assigned yet. Nonetheless soil texture appear prominently sandy and thus C_1 values can be considered a reliable index of the actual HC. Mean values of C_1 for each inoculation sites are reported in

figure 18. In all the cases only thicker crusts were chosen for the assay. On the site inoculated in 2005, a thin immature crust and a physical crust were also tested and compared with a mature crusts in order to be able to investigate the contribution of a non-biological coverage to soil sorptivity.

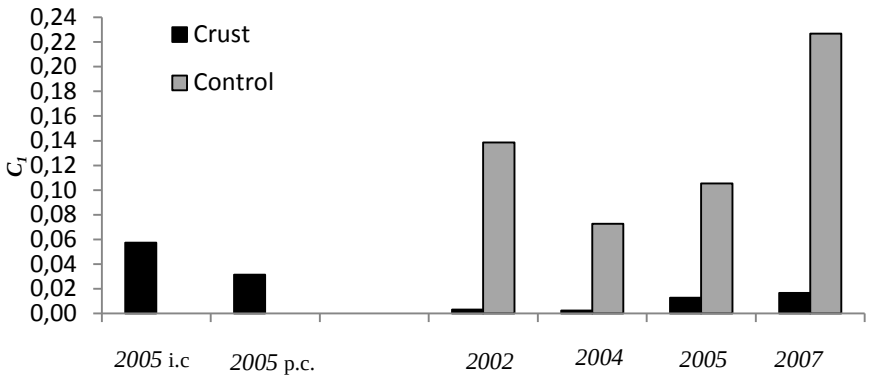

Fig 18. *Parameters C_1 for the four inoculation sites. In all the case a same suction rate was set on the tensiometer for the measurements of both the crusts and the bare soil to have comparable data. In all the cases spots covered by thick mature crust were chosen for the assays and in 2005 site both an immature thin crust and a physical crust covered spot were assayed. Abbreviations:* i.c., *immature crust;* p. c. *physical crust.*

From these data it is clearly visible how soil coverage has an effect on hydraulic conductivity compared to bare soil, clearly reducing water entry. This was expected as water is quickly dispersed and quickly lost by evaporation in sandy soils while with crust development the moisture is retained, leading to almost a complete reduction of the infiltration rate in 2002 and 2004 sites which are the first inoculated spots. Most likely this is due to the longer period of time in which these crusts had the time to grow and spread, compact the soil upper layer, compared to 2005 and 2007 sites where infiltration rate values are slightly higher.

2005 physic crust and immature crust coverage appeared a less effective barrier to water entry, compared to biological coverage.

5.1.2 Carbohydrate quantification. After HC measurements inoculation sites were sampled and total carbohydrates and high molecular weight carbohydrate (namely EPS) per gram of sediment were evaluated in laboratory conditions both for the crusts and for the underlying soil following the method described in paragraph 2.1.2. As shown in figure 19 extracellular carbohydrates resulted concentrated almost entirely in the crusts, while the amount in the underlying soil resulted minimal or no detectable.

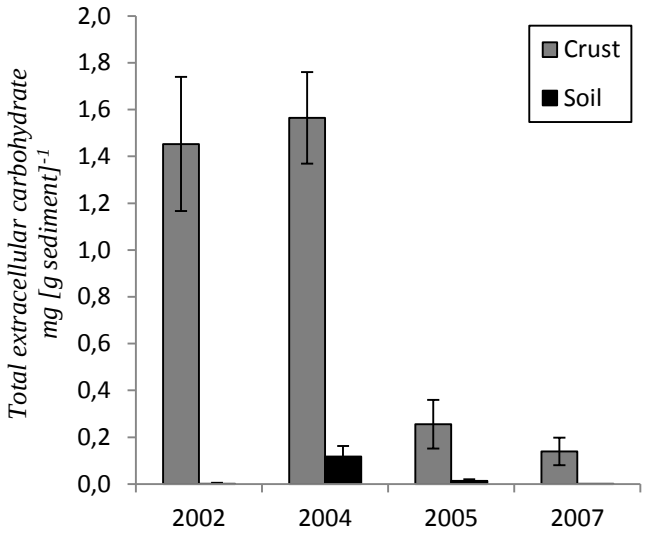

Fig. 19. *Total crust and soil carbohydrate concentrations (expressed as mg [g sediment]$^{-1}$)*

Interestingly 2002 and 2004 inoculation sites show higher amount of extracellular carbohydrates than 2005 and 2007 sites which share a similar lower amount. This suggests an overall increase of carbohydrates secretion in correlation with inoculation time, following crust development.

Plotting C_1 values related to hydraulic conductivity and carbohydrate contents evaluated for a significative number of spots covering all the inoculation sites (figure 20), non-negligible correlations are observable. Both total carbohydrate content (R^2=0,455) and EPS contents (R^2=0,446) show to affect hydraulic conductivity.

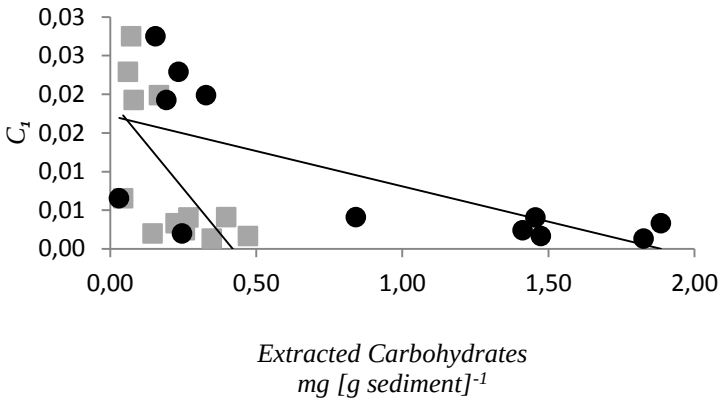

Fig 20. *Correlation between C_1 value and total carbohydrate content (expressed as mg [g sediment]$^{-1}$) (●) and C_1 value and ethanol-precipitated carbohydrates (■).*

The extent is similar to the one observed investigating on north American natural BSC (see chapter 4) regarding total carbohydrates while here also EPS seem to contribute in similar extent to affect crust relations with water an active role.

In both cases the correlation shows to be negative, suggesting that the minor water penetration is likely to be dependent from extracellular carbohydrates (both total and EPS) acting retaining water.

5.1.3 Pigment extraction and quantification. Applying pigment extraction method described in the paragraph 2.4, aliquots of samples were treated with 90% acetone and pigment quantification was obtained normalizing corrected absorbances on the dry weight. Chlophyll a, scytonemin and carotenoids contents where thus obtained (figure 21).

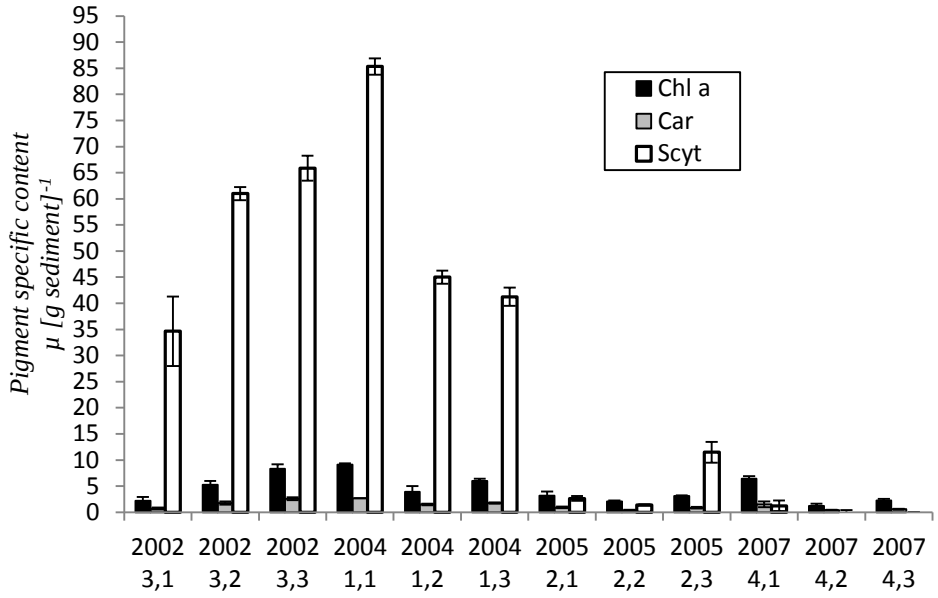

Fig. 21. *Specific pigment content (expressed as μg [g sediment]$^{-1}$) obtained analyzing 12 different spots covering the four inoculation sites.* Chla, *chlorophyll a;* Car, *carotenoids;* Scyt, *scytonemin*.

No correlation between inoculation year and phototrophic abundance was observed as within the same inoculation sites chlorophyll a amount per gram of crust shows significative variations. Scytonemin content also showed to be variable, although its maximum can be observed for 2004 inoculation sites, in the maximum amount of chlorophyll a was also revealed, thus suggesting cyanobacteria as their main producers. Higher carbohydrate amounts were also revealed in 2004 sites thus strongly supporting this latest hypothesis.

Normalizing scytonemin amounts on chlorophyll a amounts (figure 22), an increase of scytonemin is observable from the most recent inoculated sites (2007) to the oldest inoculation sites (2002) while no such variation in amount can be observed for carotenoids. This might as well be interpreted as an adaptation strategy. The scytonemin synthesis increase in the years as scytonemin-producers organisms colonize the spots previously inoculated with *Microcoleus sp.*, which devoids of protective pigments.

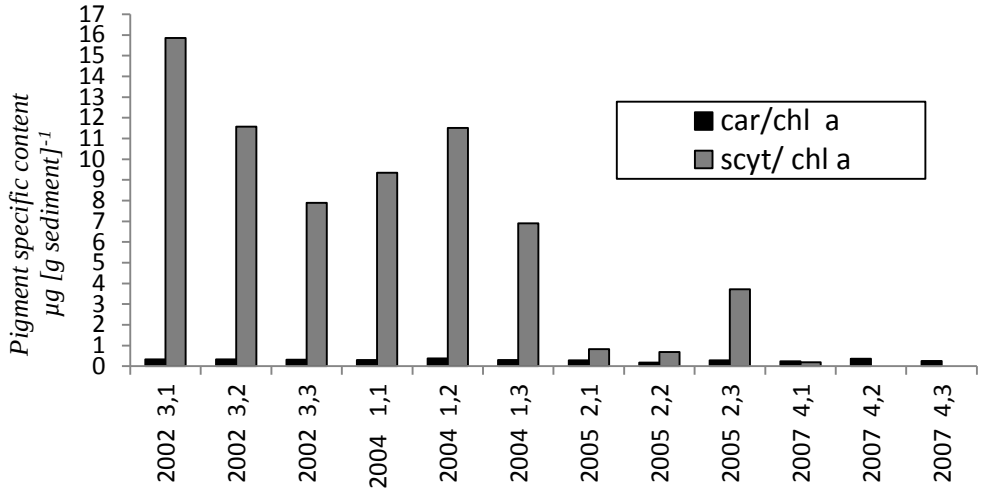

Fig.22. *Specific pigment content (expressed as µg pigment [µg chlorophyll a]$^{-1}$) obtained analyzing 12 different spots covering the four inoculation sites. Chla, chlorophyll a; Car, carotenoids; Scyt, scytonemin.*

Phenotypical analysis and specie abundances are needed to ultimately process this latest data set.

5.1.4 Exopolysaccharide hygroscopy test. The capacity of lowering water flow rate in the soil is surely enhanced by the hygroscopic properties of polysaccharides. Water captation and retention of small quantities of moisture, e.g. atmospheric humidity, becomes essential for survival in drought environments.

Hygroscopy of the crusts and finally the contribute of extracellular polysaccharides were evaluated in laboratory conditions, drying samples in oven at 37°C overnight until they completely lose their moisture and then exposing homogenized BSC and underlying soil to atmospheric humidity and observing increase in weight.

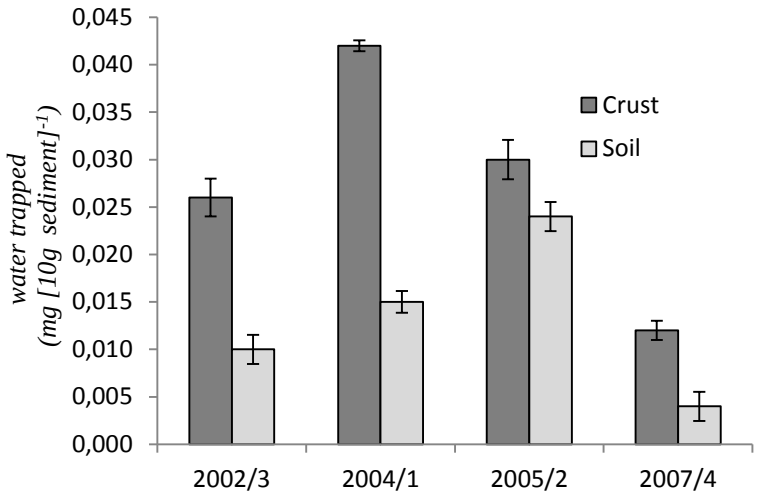

Fig.23. *Water trapped from atmospheric humidity (expressed as mg [10g sediment]$^{-1}$) due to atmospheric humidity by crust sediments from the four inoculation sites. Measurements were repeated at least three times and SD are reported in the graph.*

Homogenized crusts resulted in all cases more active in water captation than the soil on which they grew confirming their capacity of maintaining the moisture even with a limited humidity source.

The role of polysaccharides in such a phenomenon was investigated extracting exopolysaccharides and then repeating the dry test. In this case the extraction protocol was slightly modified than the treatment described in paragraph 2.1.2. The aliquots of 10g crust used for the control dry test were treated with 0.1M EDTA solution for 2h at room temperature. Afterwards samples were centrifuged at 6000 x g for 20'. The treatment was repeated four times and its effectiveness was confirmed performing phenol sulfuric assay on the extracts (data not shown). Water captation capacity of crust samples deprived of EPS was again compared with water captation capacity of the underlying soil. The result of the subsequent dry test is summarized in figure 24.

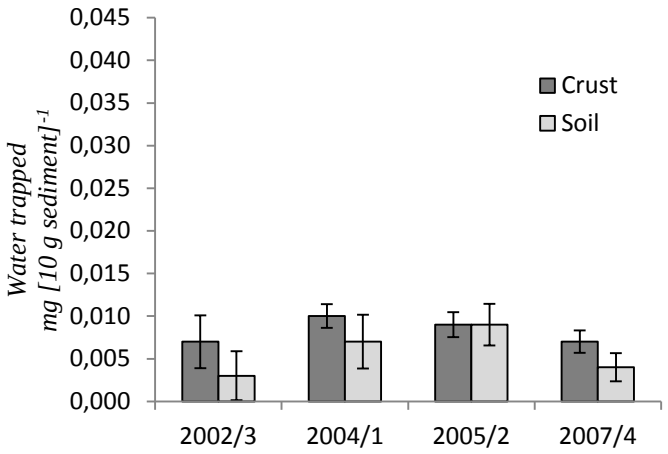

Fig.24. *Water trapped from atmospheric humidity (expressed as mg[10g sediment]$^{-1}$) due to atmospheric humidity by crust sediments from the four inoculation sites treated for EPS extraction. Experimental procedure was repeated at least three times and SD are reported in the graph.*

In this case the difference between water trapped by sand and homogenized biological soil crusts appear sensitively decreased suggesting a reduced capacity to accumulate the moisture.

5.1.5 First conclusions ad future aims. These first data confirmed the role played by exopolysaccharides in artificial BSC relationship with water, indicating the development of artificial BSC as a and suggest the need of deeper investigations to evidence marked differences or similarities with natural BSC.

The microbial composition and abundance in the sampling areas will surely help defining the contribution of cyanobacteria and to evidence the share of any other phyla in soil stabilization and EPS production.

The relations between EPS and HC will be investigated determining the composition of the polysaccharidic fraction of the soil matrix, to assess whether the differences in infiltration rates between the inoculation sites can be ascribed to significant differences in the Monosaccharidic composition.

6. Conclusions and future perspectives.

The results obtained pointed out and confirmed the non-negligible role played by microbial exopolysaccharides in extreme environments microbial colonization.

Our data point out the high complexity in terms of sugar composition that cyanobacterial-secreted polysaccharides can present, and that was observed both in the case of lithoic exposed surfaces colonization (paragraphs 3.3.3) and in desertic environments colonization (paragraph 2.2.3). This complexity in the first case is most likely an adaptation strategy to increase the number possible chemical interactions with the substrate (hydrophobic polymers) and with other organisms, which benefit from the stabilization activity of the matrix and from the trophic source it represents. The five isolated strains showed affinity with metal divalent cation calcium, magnesium and iron (II) mostly thanks to their negatively charged fractions (paragraph 3.3.4). In the case of Biological Soil Crusts (BSCs) the extracted exopolysaccharide complexity could also come from the contributes to other organisms which are known to secrete exopolysaccharides, e. g. microfungi and micro-green algae. In the case of North American desert sites, the amount of polysaccharides per gram of soil resulted correlated to chlorophyll a content (paragraph 4.3.2), thus suggesting a main apport given by cyanobacteria and micro-green algae in sugar synthesis.

This correlation between chlorophyll a and sugar content was not observed in the case of artificial chinese BSC, thus suggesting the contribution of other organisms in the soil matrix synthesis. A compositional analysis of extracted polysaccharides from the sample from Kubuqui desert is needed to observe the level of complexity and compare it to the data related to North American deserts.

Cyanobacterium *Microcoleus vaginatus* is a pioneer of desertic soils which was also used to inoculate the soil in Kubuqui desert. Its apport is essential for the crust development as it creates a layer rich in trophic sources which is thereafter colonizable by other organisms both photothropic and eterothrophic. The analysis of released polysaccharides from axenic culture of *Microcoleus vaginatus* will be useful to evidence a first contribute to the polysaccharidic matrix composition, which is expected to change at least in its internal sugar ratio, as new organisms are recruited and give their contribute.

Size fractions of extracellular polysaccharides will be also considered in the future investigations. In North American deserts hydraulic conductivity showed to be affected by the amount of carbohydrates per gram of dry weight but the correlation between hydraulic conductivity and ethanol-precipitable carbohydrates appeared sensibly weaker (paragraph 4.3.2). The contribute of high weight polysaccharides resulted negligible, while low weight carbohydrate contribute is highlighted, suggesting for them a proper role in the pore structuration of the crusts.

On the contrary, in the case of Kubuqui desert artificial crusts, the correlation between ethanol-precipitable carbohydrates and hydraulic conductivity appeared sensible, suggesting further studies to elucidate this different behaviour.

Investigations will be carried forward with methods such as size exclusion chromatography (SEC) and the results will be used to try to explain the different responses observed in the crust relations with water in terms of contribution of polymers with different molecular masses.

The tension infiltrometer proved to be a reliable instrument to measure hydraulic conductivity of BSC both on the field and in laboratory conditions.

Hydraulic conductivity resulted strongly affected by soil texture (paragraph 4.3.2): in particular is enhanced by sand content and reduced by silt and clay contents. The contribute of exopolysaccharides is however noticeable when they are removed using the non-destructive method developed during this PhD (paragraphs 2.1.4 and 4.1.7). The decrease in hydraulic conductivity measured after the treatment is realistically related to a change in pore system distribution which generates new obstacles for the water flow. In this case further microscopical investigations will be carried out in order to identify the actual changes in water circulation patterns, indirectly stating exactly the weight of the exopolysaccharides in this phenomenon.

The protection against high solar irradiation resulted also very important both for cyanobacterial stone biofilms and for BSC.

In the first case the five isolated strains showed to adapt to UV irradiation so to improve their tolerance even against irradiation in the wavelength of UVC.

This resulted, at least in part, mostly thanks to the synthesis of scytonemin and MAAs (paragraph 3.3.5 and 3.3.6).

Scytonemin contents were also evaluated in Kubuqui desert crusts (paragraph 5.1.3). If scytonemin content is normalized on chlorophyll a content an increase from 2007

sites to 2002 sites is observable. This suggest a growth in time of scytonemin levels as microorganisms adapt to survive to sunlight.

All the investigations carried out on BSC from hot environments will be extended to BSC from cold deserts, in particular collected around the scientific settlement located in Ny-Ålesund, Svalbaard *archipelago*, in collaboration with Research National Council (CNR), Florence. The objective is to have a full comparison not only between natural and artificial crusts, but also between communities living in different environmental conditions and evidence common and peculiar adaptation strategies.

These results obtained encourages in going further with these studies as they can lead to future control strategies on biofilm formations. Knowing deeply the key processes at the basis of the great ecological flexibility of cyanobacteria and phototrophic biofilms can suggest where and how to intervene.

On one hand further studies it could lead to elaborate countermeasures to avoid microbial spread on lithic artworks; on the other hand it could lead to obtain optimal conditions to enhance BSC spread, as a valid tool to counteract global desertification.

References

(1) Donlan RM (2002) Biofilms: Microbial life on surfaces. *Emerging infectious deseases*, 8 (9):881-890.

(2) Denkhaus E, Meisen S, Telgheder U, Wingender J (2007) Chemical and physical methods for characterisation of biofilms. *Microchimica Acta*, Springer, 158 (1-2): 1-27

(3) Zhang X Q, Bishop P L, Kupferle M J (1998). Measurement of polysaccharides and proteins in biofilm extracellular polymers. *Water Sci Technol,* 37: 345-348.

(4) Fletcher M, Loeb GI (1979). Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Appl Environ Microbiol*, 37: 67–72.

(5) Rosenberg M, Bayer E A, Delarea J, Rosenberg E (1982). Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *Appl Environ Microbiol*, 44: 929–937.

(6) Bashan Y, Levanony H (1988). Active attachment of *Azospirillum brasilense* Cd to quartz sand and to a light-textured soil by protein bridging. *J Gen Microbiol*, 134: 2269–2279.

(7) Danielsson A, Norkrans B, Bjornsson A (1977). On bacterial adhesion – the effect of certain enzymes on adhered cells in a marine *Pseudomonas* sp. *Bot Marina*, 20: 13–17.

(8) Brading M G, Jass J, Lappin-Scott H M (1995). Dynamics of bacterial biofilm formation. In: Lappin-Scott H M, Costerton J W (eds.). *Microbial biofilms*. Cambridge University Press, 46–63.

(9) Tolker-Nielsen T, Molin S (2000). Spatial organization of microbial biofilm communities. *Microb Ecol*, 40: 75–84.

(10) James G A, Beaudette L, Costerton J W (1995). Interspecies bacterial interactions in biofilms. *Journal of Industrial Microbiology*, 15: 257–262.

(11) Costerton J W, Cheng K J, Geesey G G, Ladd T I, Nickel J C, Dasgupta M, Marrie T J (1987). Bacterial Biofilms in Nature and Disease. *Annual Review of Microbiology*, 41: 435-464.

(12) Kühl M, Jørgensen B B (1992). Microsensor Measurements of Sulfate Reduction and Sulfide Oxidation in Compact Microbial Communities of Aerobic Biofilms. *Appl. Environ. Microbiol*. , 58: 1164-1174.

(13) Jones H C, Roth I L, Saunders W M III (1969). Electron microscopic study of a slime layer. *J Bacteriol*, 99: 316–325.

14) Costerton J W, Geesey G G, Cheng K-J (1978). How bacteria stick. *Sci Am* , 238: 86–95.

(15) Characklis W G (1973). Attached microbial growths. II. Frictional resistance due to microbial slimes. *Water Res*, 7: 1249–1258.

(16)Lawrence J R, Neu T R (2003). Microscale analyses of the formation and nature of microbial biofilm communities in river systems. *Rev environ Sci Bio Tech*, 2: 85.

(17) De Kievit T R, Gillis R, Marx S, Brown C, Iglewski B H (2001). Quorum-Sensing Genes in *Pseudomonas aeruginosa* Biofilms: Their Role and Expression Patterns. *Applied and Environmental Microbiology*, 67 (4): 1865-1873.

(18) Guzzon A, Bohn A, Diociaiuti M, Albertano P (2008). Cultured phototrophic biofilms for phosphorus removal in wastewater treatment. *Water Research*, 42 (16): 4357-4367.

(19) Nielsen P H, Jahn A, Palmgren R. (1997). Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology*, 31 (1): 11-19.

(20) Geesey G G (1982). Microbial exopolymers: ecological and economical considerations. *ASM news*, 48: 9-14.

(21) Characklis W G, Wilderer P A (1989) (glossary). In: Characklis W G, Wilderer P A. *Structure and Function of Biofilms*, Wiley, Chichester, 369-371.

(22) Flemming H-C, Neu T R, Wozniak D J (2007). The EPS Matrix: The "House of Biofilm Cells". *Journal of Bacteriology*, 189 (22): 7945-7947.

(23) Allison D G, Sutherland I W, Neu T R (2003). EPS: what's an acronym? p. 381–387. In: Mc Bain A, Allison D, Brading M, Rickard A, Verran J, Walker J (eds.). *Biofilm communities: order from chaos?*. Bio- Line, Cardiff, United Kingdom.

(24) Platt R M, Geesey G G, Davis J D, White D C (1985). Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. *an. J. Microbiol.*, 31(8): 675–680.

(25) Frølund B, Palmgren R, Keiding K (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research*, 30 (8): 1749-1758.

(26) Neu T R (1996). Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol rev*, 60: 151-166.

(27) Takeda, Minoru, Nakano F, Nagase T, Iohara K, Koizumi J (1998). Isolation and Chemical Composition of the Sheath of *Sphaerotilus natans*. *Bioscience, Biotechnology and Biochemistry*, 62: 1138-1143.

(28) Burns R G (1989). Microbial and enzymatic activities in soil biofilms. In: Characklis W G, Wilderer PA (eds.). *Structure and functions of biofilms*. Wiley, Chichester, 333-349.

(29) Jahn A, Nielsen P H (1998). Cell Bimass and exopolymer composition in sewer biofilms. *Wat. Sci. Technol.*, 37: 17-24.

(30) Binet R, Létoffé S, Ghigo J M, Delepelaire P, Wandersman C (1997). Protein secretion by Gram-negative bacterial ABC exporters-a review. *Gene*, 192 (1): 7-11.

(31) Filloux A, Michel G, Bally M (1998). GSP-dependent protein secretion in gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.*, 22: 177-198.

(32) Lorenz M G, Wackernagel W (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev*., 58 (3): 563–602.

(33) Beveridge T J, Makin S A, Kadurugamuwa J L, Li Z (1997). Interactions between biofilms and the environment. *FEMS Microbiol. Rev.*, 20: 291-303.

(34) Li Z, Clarke A J, Beveridge T J (1998). Gram-Negative Bacteria Produce Membrane Vesicles Which Are Capable of Killing Other Bacteria. *Journal of Bacteriology*, 180 (20): 5478-5483.

(35) Hedges J I (1988). Polymerization of humic substances in natural environments. In: Frimmel F H, Christman R F (eds.) *Humic substances and their role in the environment*. Wiley, Chichester, 45-58.

(36) Costerton J W, Irvin R T, Cheng K J (1981). The bacterial glycocalyx in nature and disease. *Annu. Rev. Microbiol.*, 35: 299-324.

(37) Roberts I S (1996). The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol*., 50: 285-315.

(38) Nielsen P H, Jahn A (1999). Extraction of EPS. In: Wingender J, Neu T R and Flemming H-C (eds.) *Microbial Extracellular Polymeric Substances*. Springer-Verlag, Berlin, Germany.

(39) Flemming H-C, Wingender J (2003). The crucial role of extracellular polymeric substances in biofilms. In: Wuertz S, Bishop PL, Wilderer PA (eds.) *Biofilm in Wastewater Treatment: an interdisciplinary approach*. IWA Publishing, London, 78.

(40) Sutherland I W (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, 147: 3-9.

(41) Sutherland I W (1969). Structural studies on colanic acid, the common exopolysaccharide found in the enterobacteriaceae, by partial acid hydrolysis. Oligosaccharides from Colanic Acid. *Biochem. J.*, 115, 935.

(42) Pereira S, Zille A, Micheletti E, Moradas-Ferreira P, De Philippis R, Tamagnini P (2009). Complexity of cyanobacterial exopolysaccharides: composition, structures,

inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiology Reviews*, 33: 917-941.

(43) Thu B, Skjåk-Br[aelig]k G, Micali F, Vittur F (1997).The spatial distribution of calcium in alginate gel beads analysed by synchrotron-radiation induced X-ray emission (SRIXE). *Carbohydrate Research*, 297 (2): 101-105.

(44) LeChevallier M W, Cawthon C D, Lee R G (1988). Inactivation of biofilm bacteria. *Appl Environ Microbiol*, 54 (10): 2492-2499.

(45) Foley I, Gilbert P (1996). Antibiotic resistance of biofilms. *Biofouling*, 10 (4): 331 – 346.

(46) Weiner R, Langille S, Quintero E (1995). Structure, function and immunochemistry of bacterial exopolysaccharides. *J. Ind. Microbiol*., 15: 339-346.

(47) De Philippis R, Vincenzini M (1998). Exocellular polysaccharides from cyanobacteria and their possible applications, *FEMS Microbiology Reviews*, 22: 151-175.

(48) Caldwell D E, Wolfaardt G M, Korber D R, Lawrence J R (1996). Cultivation of microbial consortia and communities. In: Hurst C J, Knudson G R, Mc Inerney M J, Stetzenbach L D, Walker M V (eds) *Manual for environmental Microbiology*. American Society for Microbiology, 79-90.

(49) Hebbar K P, Gueniot B, Heyraud A, Colin-Morel P, Heulin T, Blandreau J, Rinaudo M (1992). Characterization of exopolysaccharides produced by rhizobacteria. *Appl. Microbiol. Biotechnol*., 38: 248-253.

(50) Karapanagiotis N K, Rudd T, Sterritt R M, Lester J N (1989). Extraction and characterization of extracellular polymers in digested sewage sludge. *J Chem Tech Biotecnol*, 44: 107-120.

(51) Zayas J F (1997). Functionality of proteins in food. Springer, Berlin, Heidelberg, New York

(52) Read R R, Costerton J W (1987). Purification and characterization of adhesive

exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. *Can. J. Microbiol.,* 33: 1080-1090.

(53) Christensen B E, Kjosbakken J, Smidsrød O (1985). Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. strain NCMB 2021. *Appl. Environ. Microbiol*., 50: 837-845.

(54) Underwood G J C, Paterson D M, Parkes R J (1995). The measurement of microbial carbohydrate exopolymers from interstitial sediments. *Limnol. Oceanogr*., 40: 1243–1253.

(55) Sato T, Ose Y (1984). Floc-forming substances extracted from activated sludge with ammonium hydroxide and EDTA solutions. *Wat Sci Tech,* 17: 517-528.

(56) Grotenhuis J T C, Smit M, Van Lammeren A A M, Stams A J M, Zehnder A J B (1991). Localization and quantification of extracellular polymers in methanogenic granular sludge. *Appl. Microbiol. Biotechnol.,* 36: 115-119.

(57) Platt R M, Geesey G G, Davis J D, White D C (1985). Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. *Can. J. Microbiol*., 31(8): 675–680.

(58) May E, Lewis F J, Pereira S, Tayler S, Seaward M R D, Allsopp D (1993). Microbial deterioration of building stone: a review. *Biodeterioration abstracts,* 7: 109-123.

(59) Ortega-Morales O, Guezennec J, Hernandez-Duque G, Gaylarde C C, Gaylarde P M (2000). Phototrophic biofilms on ancient Mayan buildings in Yucatan, Mexico. *Curr.Microbiol*., 40: 81-85.

(60) Braams J (1992). Ecological studies on the fungal microflora inhabiting historical sandstone monuments. PhD thesis, Oldenburg, Germany.

(61) Arino X, Saint-Jimenez C (1996). Factors affecting the colonization and distribution of cyanobacteria, algae and lichens in ancient mortars. In: Riederer J (ed.) *Proceedings of the eight International Congress on Deterioration and*

Conservation of stone, Rathgen-Forschungslabor, Berlin, Germany, (2): 725-731.

(62) Grant C, Bravery A F (1985). A new method for assessing the resistance of stone to algal disfigurement and the efficiency of the chemical inhibitors. In: Felix G (ed.) *Proceedings of the Fifth International Congress on Deterioration and Conservation of Stones*. Presses Polyechniques Romandes, Lousanne. (2): 663-674

(63) de la Torre M A, Gomez- Alarcon G, Melgarejo P, Saiz-Jimenez C. Fungi in weathered sandstone from Salamanca cathedral, Spain. *The Science of The Total Environment*, 107: 159-168.

(64) Crispim C A, Gaylarde C C (2005). Cyanobcteria and Biodeterioration of Cultural Heritage: A review. *Microbial Ecol*, 49, 1-9.

(65) Gillat J W (1991). The need of an antifungal and antialgal additives in high performance coatings. *Surface coatings Internat*, 74: 6-12.

(66) Ortega-Calvo J J, Hernandez-Marine H, Saiz-Jimenez C (1991). Biodeterioration of building stones by cyanobacteria. *Int. Biodeter.*, 28: 165-186.

(67) Warscheid T, Petersen K, Krumbein WE (1989) Procedures and methods for the assessment of microbiological influences on stone decay. In: *Technology and European Cultural Heritage*. Commission of the European communities Bologna. National Research Council, Brussel, 489-492

(68) Warscheid T, Becker TW, Braams J (1993). Studies on the temporal development of microbial infection of different types of sedimentary rocks and its effect on the alteration of the physico-chemical properties in building materials. In: Thiel M-J, (ed.), *Conservation of Stone and Other Materials*, RILEM, 1: 303–310

(69) Urzì, C., Criseo, G., Krumbein, W. E., Wollenzien, U., & Gorbushina, A. A. (1993). Are colour changes of rocks caused by climate, pollution, biological growth, or by interactions of the three? In: Thiel M-J (Ed.), *Conservation of stone and other materials*, London, E & FN Spon, 279-286.

(70) Urzì C, Krumbein W E, Warscheid T (1992). On the question of biogenic colour changes of mediterranean monuments (coating - crust - microstromatolite - patina - scialbatura - skin - rock varnish). In: Decrouez D, Chamay J, Zezza F (eds.),

Proceedings of the 2nd International Symposium on The conservations of the monuments in the Mediterranean Basin, Musee d'Art et d'Histoire Naturelle, Genève, 397-420.

(71) Warscheid T, Braams J (2000). Biodeterioration of stone: a review. *International Biodeterioration & Biodegradation*, 46: 343-368.

(72) Lazzarini L, Salvadori O (1989). A reassessment of the formation of the patina called scialbatura. *Studies in Conservation*, 34: 20-26.

(73) Pinna D (1993). Fungal physiology and the formation of calcium oxalate films on stone monuments. *Aerobiologia*, 9: 157-167.

(74) Bethelin J (1983). Microbial weathering processes. In: Krumbein W E (ed.) *Microbial geochemistry*. Blacwell Scientific Publications, Oxford, 223-262.

(75) Keller WD (1957). *Principles of Chemical Weathering*. Lucas Brothers Publishing, Columbia, Missouri.

(76) Vourinen A, Manterc-Alhonen S, Uusinoka R, Alhonen P (1981). Bacterial Weathering of Rapakivi granite. *J Geomicrobiol*, 2: 317-325.

(77) Callot G, Maurette M, Pottier L, Dubois A (1987). Biogenic etching of microfractures in amorphous and crystalline silicates. *Nature*, 328: 147-149.

(78) Beveridge T J, Murray R G E (1976). Uptake and retention of metals by cell walls of Bacillus subtilis. *Journal of Bacteriology*, 127: 1502-1518.

(79) Iskandar I K, Syers J K (1972). Metal-complex formation by lichen compounds. *Journal of Soil Science*, 23: 255-265.

(80) Lundgren D G (1989). Biotic and abiotic release of inorganic substances exploited by bacteria. In: Poindexter J S, Leadbetter E R (eds.) *Bacteria in Nature*, Plenum Press, New York. 3:293.

(81) Decho A W, Visscher P T, Reid R P (2005). Production and cycling of natural microbial exopolymers (EPS) within a marine stromatolithe. *Palaeo*, 219: 71–86.

(82) Geesey G G, Jang L (1989). Interactions between metal ions and capsular polymers. In: Beveridge T J, Doyle R J (eds.) *Metal ions and bacteria*. Wiley, New York, 325–357.

(83) Decho A W, Visscher P T, Ferry J, Kawaguchi T, He L, Przekop K M, Norman R S, Reid R P (2009). Autoinducers extracted frommicrobialmats reveal a surprising diversity ofN-acylhomoserine lactones (AHLs) and abundance changes that may related to diel pH. *Environ. Microbiol*., 11: 409–420.

(84) Dupraz C, Visscher P T (2005). Microbial lithification in marine stromatolites and hypersaline mats. *Trends Microbiol*., 13: 429–438.

(85) Brown I, Mummey D, Sarkisova S, Allen C, McKay D S (2005). Iron-Tolerant Cyanobacteria as an Effective Tool to Study Early Evolution of Life and the Development of Biosignatures. 36th *Lunar and Planetary Science meeting*, League City, TX.

(86) Proteau P J, Gerwick W H, Garcia-Pichel F, Castenholz R W (1993). The structure of scytonemin, an ultraviolet sunscreen pigment from the sheath of cyanobacteria. *Experimentia*, 49: 825-829.

(87) Garcia-Pichel F, Castenholz R W (1991). Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. *Journal of Phycology*, 27 (3): 395–409.

(88) Garcia-Pichel F, Sherry N D, Castenholz RW (1992). Evidence for a UV sunscreen role of the extracellular pigment scytonemin in the terrestrial cyanobacterium *Chlorogloeopsis* sp. *Photochem. Photobiol*. 56: 17-23.

(89) Portwich A, Garcia-Pichel, F (2000). A Novel Prokaryotic UVB Photoreceptor in the Cyanobacterium *Chlorogloeopsis* PCC 6912. *Photochemistry and Photobiology*, 71 (4) 493-498

(90) Böhm G A, Pfleiderer W, Böger P, Scherer S (1995). Structure of a novel oligosaccharide- mycosporine-amino acid ultraviolet A/B sunscreen pigment from the terrestrial cyanobacterium *Nostoc commune*.*J Biol Chem*, **270**: 8536–8539.

(91) Belnap J (2006). The potential roles of biological soil crusts in dryland hydrologic cycles. *Hydrol. Process.*, 20: 3159–3178.

(92) Belnap J, Kaltenecker J H, Rosentreter R, Williams J, Leonard S, Eldridge D (2001). Biological Soil Crusts: Ecology and Menagement. *Technical Reference*, 1730-2

(93) Belnap J (2003). Comparative structure of physical and biological soil crusts. In: Belnap J, Lange O L (eds.). *Biological Soil Crusts: Structure, Function, and Management*. Springer-Verlag, Berlin, 177–191.

(94) Belnap J, Gillette D A (1997). Disturbance of biological soil crusts: impacts on potential wind erodibility of sandy desert soils in southeastern Utah, USA. *Land Degradation and Development*, 8: 355-362.

(95) Belnap J, Gillette D A (1998). Vulnerability of desert soil surfaces to wind erosion: impacts of soil texture and disturbance. *Journal of Arid Environments*, 39: 133-142.

(96) Kidron G J, Yaalon D H, Vonshak A (1999). Two causes of runoff initiation on microbiotic crusts: hydrophobicity and pore clogging, *Soil Science*, **164**: 18–27.

(97) Eldridge D J, Zaady E, Shachak M (2000). Infiltration through three contrasting biological soil crusts in patterned landscapes in the Negev, Israel. *Catena*, 40(3): 323-336

(98) Greene R S B, Tongway D J (1989). The significance of (surface) physical and chemical properties in determining soil surface condition of red-earths in rangelands. *Aust. J. Soil Res.*, 27: 213-25.

(99) Eldridge D J (1993). Cryptogams, vascular plants, and soil hydrological relations: some preliminary results from the semiarid woodlands of eastern Australia. *Great Basin Naturalist*, 53: 48–58.

(100) Decho AW (1990). Microbial exopolymers secretions in ocean environments: Their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Annu. Rev.*, 28: 73-153.

(101) Dubois M, Gilles K A, Hamilton J K, Rebers P A, Smith F (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.*, 28 (3): 350–356.

(102) de Brouwer J F C, Stal L J (2001). Short-term dynamics in microphytobenthos distribution and associated extracellular carbohydrates in surface sediments of an intertidal mudflat. *Mar Ecol Prog Ser* , 218: 33–44..

(103) Kirkham M B (2005). *Principles of soil and plant water relations*. Elsevier Academic Press, Burlington, MA, 145-172.

(104) Zhang R (1997). Determination of soil sorptivity and hydraulic conductivity from the disk infiltrometer. *Soil Sci. Soc. Am. J.*, 61: 1024-1030.

(105) Garcia-Pichel F, Castenholz R W (1993). Occurrence of UV-Absorbing, Mycosporine-Like Compounds among Cyanobacterial Isolates and an Estimate of Their Screening Capacity. *Appl Environ Microbiol*, 163-169.

(106) Vernon L P (1960). Spectrophotometric determination of chlorophylls and phaeophytins in plant extracts. *Anal. Chem.*, 32: 1144–1150.

(107) Britton G (1985). General carotenoid methods. *Methods Enzymol*., 111: 113–158.

(108) Böhm G A, Pfleiderer W, Böger P, Scherer S (1995). Structure of a novel oligosaccharide-mycosporine-amino acid ultraviolet A/B sunscreen pigment from the terrestrial cyanobacterium *Nostoc commune*. *J. Biol. Chem.*, 270: 8536–8539.

(109) de la Torre AM, Gomez-Alarcon G, Vizcaino C, Garcia M T (1993). Biochemical mechanisms of stone alteration carried out by filamentous fungi living in monuments. *Biogeochemistry*, 19: 129-147.

(110) Gaylarde C C, Morton L H G (1999). Deteriogenic biofilms on buildings and their control: a review. *Biofouling*, 14(1): 59-74.

(111) Wakefield R D, Jones M S (1998). An introduction to stone colonizing micro-organisms and biodeterioration of building stone. *Q J Eng Geol*, 31: 301–313.

(112) Warscheid T (1996). Biodeterioration of stones: analysis, quantification and evaluation. *Proceedings of the 10th International Biodeterioration and Biodegradation Symposium*, Dechema-Monograph, 133, Dechema, Frankfurt, 115-120.

(113) Lamenti G, Tiano P, Tomaselli L (2000). Biodeterioration of ornamental marble statue in the Boboli Gardens (Florence, Italy). *J Appl Phycol*, 12: 427-433.

(114) Tiano P, Accolla P, Tomaselli L (1995). Phototrophic Biodeteriogens on lithoid surfaces: an ecological study. *Microb Ecol*, 29: 299-309.

(115) Friedmann E I, Weed R (1987). Microbial trace fossil formation, biogenous, and abiotic weathering in the antartic cold desert. *Science*, 236: 703-705.

(116) De Philippis R, Vincenzini M (2003). Outermost polysaccharidic investments of cyanobacteria: nature, significance and possible applications. *Recent Res.Devel. Microbiology*, 7: 13-22.

(117) Krumbein W E, Petersen K (1988). Microbial interactions with mineral materials. In: Houghton D R, Smith R N, Eggins H O W (eds.) *Biodeterioration*, 7, Elsevier Applied Sciences, London, 78-100.

(118) Garty J (1991). Influence of epilithic microorganism on the surface temperature of building walls. *Can J Bot*, 68: 1349-1353.

(119) Reddy K J, Soper B W, Tang J, Bradley R L (1996). Phenotypic variation in exopolysaccharide production in the marine, aerobic nitrogen-fixing unicellular cyanobacterium *Cyanothece* sp. *World Journal of Microbiology & Biotechnology*, 12: 311-318.

(120) Ramus J (1977). Alcian blue: a quantitative aqueous assay for algal acid and sulfated polysaccharides. *Journal of Phycology* ,13 (4): 345–348.

(121) Bar-Or Y, Shilo M (1987). Characterization of macromolecular flocculants produced by Phormidium sp. strain J-1 and by Anabaenopsis circularis PCCC 6720. *Appl. Environ. Microbiol*, 53: 2226-2230.

(122) Volesky B, May-Phillips H A (1995). Biosorption of heavy metals by *Saccharomyces cerevisiae. J. Appl. Microbiol. Biotechnol.*, 42: 797-806.

(123) Micheletti E, Pereira S, Mannelli F, Moradas-Ferreira P, Tamagnini P, De Philippis R (2008). Sheathless Mutant of Cyanobacterium *Gloeothece* sp. Strain PCC 6909 with Increased Capacity To Remove Copper Ions from Aqueous Solutions. *Appl. Environ. Microbiol*, 74 (9): 2797–2804.

(124) Rippka R, Deruelles J, Waterbury J, Herdman M, Stanier R (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol*., 111: 1-61.

(125) Khattar J I S, Singh D P, Namita Jindal, Kaur N, Singh Y, Rahi P, Gulati A (2010). Isolation and Characterization of Exopolysaccharides Produced by the **c**yanobacterium *Limnothrix redekei* PUPCCC 116. *Applied biochemistry and biotechnology*, 162 (5): 1327-1338.

(126) De Philippis R, Sili C, Paperi R, Vincenzini M (2001). Exopolysaccharide-producing cyanobacteria and their possible exploitation: a review. *Journal of Applied Phycology*, 13: 293-299.

(127) Bellezza S, Paradossi G, De Philippis R, Albertano P (2003). Cytochemical and physico-chemical characterisation of exopolysaccharides in Leptolyngbya sp. strains from Roman hypogea: looking for new approaches in conservation of stone. *Journal of Applied Phycology*, 15: 193-200.

(128) Decho AW (2000). Microbial biofilms in intertidal systems: an overview. *Cont Shelf Res*, 20: 1257-1273.

(129). Mancuso Nichols C A, Guezennec J, Bowman J P (2005). Bacterial Exopolysaccharides from Extreme Marine Environments with Special Consideration of the Southern Ocean, Sea Ice, and Deep-Sea Hydrothermal Vents: a Review. *Marine Biotechnology*, 7(4) : 253-271.

(130) Arias S, del Moral A, Ferrer M R, Tallon R, Quesada E, Béjar V (2003). Mauran, an exopolysaccharide produced by the halophilic bacterium Halomonas maura, with a novel composition and interesting properties for biotechnology. *Extremophiles*, 7: 319-326.

(131) Sutherland I W (1994). Structure-function relationships in microbial exopolysaccharides. *Biotechnol Adv*, 12: 393-448.

(132) Pierson B K, Parenteau M N (2000). Phototrophs in high iron microbial mats: microstructure of mats in iron-depositing hot springs. *FEMS Microbiology Ecology*, 32: 181-196.

(133) Brown M J, Lester J N (1982). Role of bacterial extracellular polymers in metal uptake in pure bacterial culture and activated sludge - I. Water Res 16: 1539-1548.

(134) de Kerchove A J, Menachem M. Calcium and Magnesium Cations Enhance the Adhesion of Motile and Nonmotile *Pseudomonas aeruginosa* on Alginate Films. *Langmuir*, 2008, *24* (7): 3392–3399

(135) Welch S A, Vandevivere P (1994). Effect of microbial and other naturally occurring polymers on mineral dissolution. *Geomicrobiology Journal*, 12, 227–238.

(136) Wolfaardt G M, Lawrence J R, Korber D R (1999). Function of EPS. In: Wingender J, Neu T R, Flemming H-C (eds.) *Microbial Extracellular Polymeric Substances*. Springer-Verlag, Berlin, 171-195.

(137) Chen J-H, Lion LW, Ghiorse W C, Shuler M L (1995). Mobilization of adsorbed cadmium and lead in aquifer material by extracellular polymers. *Wat Res*, 29: 421-430.

(138) MacLeod G, McKeown C, Hall A J, Russell M J (1994). *Orig.Life Evol. Biosph*., 24: 19-41.

(139) Pierson B K, Parenteau M N, Griffin B M (1999). Phototrophs in high-iron-concentration microbial mats: Physiological ecology of phototrophs in an iron-depositing hot spring. *Appl. Environ. Microbiol*., 65, 5474-5483.

(140) Pierson B K, Mitchell H K, Ruff-Roberts A L (1993). *Chloroflexus aurantiacus* and ultraviolet radiation: implications for Archean shallow-water stromatolites. *Orig. Life Evol. Biosph*., 23, 243-260.

(141) Pierson B K, Parenteau M N (2000). Phototrophs in high iron microbial mats: microstructure of mats in iron-depositing hot springs. *FEMS Microbiology Ecology*, 32: 181-196.

(142) Varnali T, Edwards H G. (2010). Iron-scytonemin complexes: DFT calculations on new UV protectants for terrestrial cyanobacteria and astrobiological implications. *Astrobiology*, 10(7): 711-716.

(143) Soule T, Garcia-Pichel F, Stout V (2009). Gene Expression Patterns Associated with the Biosynthesis of the Sunscreen Scytonemin in *Nostoc punctiforme* ATCC 29133 in Response to UVA Radiation. *J Bacteriol*. 191(14): 4639–4646.

(144) Ehling-Schulz M, Bilger W, Scherer S (1997). UV-B-Induced Synthesis of Photoprotective Pigments and Extracellular Polysaccharides in the Terrestrial Cyanobacterium *Nostoc commune*. *Journal of Bacteriology*, 179(6): 1940–1945.

(145) Oren A, Gunde-Cimerman N (2007). Mycosporines andmycosporine-like amino acids: UVprotectants or multipurpose secondary metabolites? *FEMS Microbiology Letters* , 269 (1): 1-10.

(146) Holzinger A, Roleda M Y, Lütz C (2009). The vegetative arctic freshwater green alga *Zygnema* is insensitive to experimental UV exposure. *Micron*, 40 (8): 831-838.

(147) Boeken B, Shachak M (1994). Desert Plant Communities in Human-Made Patches--Implications for Management. *Ecological Applications*, 4 (4): 702-716.

(148) Boeken B, Shachak M, Gutterman Y, Brand S (1995). Patchiness and Disturbance: Plant Community Responses to Porcupine Diggings in the Central Negev. *Ecography*, 18 (4): 410-422.

(149) Yair A (1987). Environmental effects of loess penetration into the northern Negev. *Journal of Arid Environments*, 13: 9-24.

(150) Warren S D (2003). Biological soil crusts and hydrology cycles in North American deserts. In: Belnap J, Lange O L (eds.). *Biological Soil Crusts: Structure, Function, and Management*, Springer-Verlag, Berlin, 327–337.

(151) Warren S D (2003). Influence of biological soil crusts on arid land hydrology and soil stability. In Belnap J, Lange O L (eds.) *Biological Soil Crusts: Structure,Function and Management*, Springer-Verlag, Berlin, 349-360.

(152) Loope W L, Gifford G F (1972). Influence of a soil microfloral crust on selected properties of soils under pinyon-juniper in southeastern Utah. *J. Sozl Water Conserv.*, 27: 164-167.

(153) Brotherson J D, Rushforth S R (1983). Influence of cryptogamic crusts on moisture relationships of a soil in Navajo National Monument, Arizona. *Great Basin Naturalist*, 43: 73-79.

(154) Romkens M J M, Prasad S N, Whisle F D (1990). Surface sealing and infiltration. In Anderson M G, Burt I P (eds.) *Process Studies in Hillslope Hydrology*, Wiley, New York, 127-172.

(155) Bisdom E B A**,** Dekker **L W,** Schoute J F T (1993). Water repellency of sieve fractions from sandy soils and relationships with organic material and soil structure. *Geoderma*, 56 (1-4): 105-118.

(156) Kidron G J, Yair A, Vonshak A, Abeliovich A (2003). Microbiotic Crust Control of Runoff Generation on Sand Dunes in the Negev. *Water Resources Research*, 39, Article Number 1118.

(157) Seghieri J, Galle S, Rajot J L, Ehrmann M (1997). Relationships between soil moisture and growth of herbaceous plants in a natural vegetation mosaic in Niger. *Journal of Arid Environments*, 36: 87–101.

(158). Graetz R D, Tongway D J (1986). Influence of grazing management on vegetation, soil structure and nutrient distribution and the infiltration of applied rainfall in a semi-arid chenopod shrubland. *Australian Journal of Ecology*, 11 (4): 347–360.

(159) Campbell S E (1979). Soil stabilization by prokaryotic desert crust: implications for Precambrian land biota, *Origin of Life*, 9: 335-348.

(160) Durrell L W, Shields L M (1961). Characteristics of soil algae relating to crust formation. *Transactions of the American Microscopical Society*, 80: 73–79.

(161) Mazor G, Kidron G J, Vonshak A, Abeliovich A (1996). The role of cyanobacterial exopolysaccharides in structuring desert microbial crusts, *FEMS Microbiology Ecology*, 21: 121-130.

(162) Fischer T, Veste M, Wiehe W, Lange P (2010). Water repellency and pore clogging at early successional stages of microbiotic crusts on inland dunes, Brandenburg, NE Germany. *Catena*, 80 (1): 47-52.

(163) Eldridge D J (2001). Biological soil crusts and water relations in Australia deserts. In: Belnap J, Lange O L (eds.) *Biological Soil Crusts: Structure, Function and Management*, Springer, Berlin, 315-325.

(164) Greene R S B (1992). Soil physical properties of three geomorphic zones in a semi-arid mulga woodland. *Aust.* J. *Soil Res*., 30: 55-69.

(165) Malam Issa O, Défarge C**,** Trichet J**,** Valentin **C,** Rajot J L (2009). Microbiotic soil crusts in the Sahel of Western Niger and their influence on soil porosity and water dynamics. Catena, 77 (1): 48-55

(166) Schwieger F, Tebbe C C (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology*, **64**: 4870–4876.

(167) Wang Y, Boukany P, Wang S-Q, Wang X (2007). Elastic Breakup in Uniaxial Extension of Entangled Polymer Melts. *Phys. Rev. Lett*. 99: 237801

(168) Dean W E (1974). Determination of carbonate and organic matter in calcareous sediments and sedimentary rocks by loss on ignition: comparison with other methods. *Journal of Simentary Petrology*, 44: 242-248.

(169) McKinney G (1941). Absorption of light by chlorophyll solutions. *J Biol Chem*, 140: 315–320.

(170) Carsel R F, Parrish R S (1988). Developing joint probability distributions of soil water retention characteristics. *Water Resour. Res.*, 24: 755-769.

(171) Brady N C, Weil R R (1996). *The nature and properties of soils*. 11th edition. Prentice-Hall, Inc., Upper Saddle River, New Jersey.

(172) Nagy M, Perez A, Garcia-Pichel F (2005). The prokaryotic diversity of biological soil crusts in the Sonoran Desert (Organ Pipe Cactus National Monument, AZ). *FEMS Microbiology Ecology*, 54: 233-245.

(173) Garcia-Pichel F, Johnson S L, Youngkin D, Belnap J (2003). Small-scale vertical distribution of bacterial biomass and diversity in biological soil crusts from arid lands in the Colorado Plateau. *Microbial Ecol*, 46: 312-321.

(174) Potts M (1994). Desiccation tolerance of procaryotes. *Microbiol. Rev.*, 58: 755-805.

(175) Dong H, Rech J A, Jiang H, Sun H, Buck B J (2007). Endolithic cyanobacteria in soil gypsum: Occurrences in Atacama (Chile), Mojave (United States), and Al-Jafr Basin (Jordan) Deserts. *Journal of geophysical research*, 112 (2): G02030.

(176) de la Torre J R, Goebel B M, Friedmann E I, Pace N R (2003). Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Appl. Environ. Microbiol*., 69: 3858-3867.

(177) Bear J(1972). *Dynamics of Fluids in Porous Media*. American Elsevier Pub. Co. , New York.

(178) Danin A, Dor I, Sandler A, Amit R (1998). Desert crust morphology and its relations to microbiotic succession at Mt. Sedom, Israel. *Journal of Arid Environments*, 38: 161-174.

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….anything worth doing is worth overdoing…