The Biofilm Matrix – Multitasking in a Shared Space

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**Abstract**

The biofilm matrix serves as a shared space for its cellular inhabitants, comprising a wide variety of extracellular polymeric substances (EPS), like polysaccharides, proteins, amyloids, lipids and extracellular DNA (eDNA), as well as membrane vesicles and humic-like, bacterially derived refractory substances. The EPS are dynamic in space and time and its components interact in complex ways, fulfilling various functions: to stabilize the matrix, acquire nutrients, retain and protect eDNA or exoenzymes, or offer sorption sites for ions and hydrophobic substances. The retention of exoenzymes effectively renders the biofilm matrix an external digestion system influencing the global turnover of biopolymers. Physicochemical and biological interactions and environmental conditions enable biofilm systems to morph into films, micro- and macro-colonies, ridges, ripples, columns, pellicles, bubbles, mushrooms and suspended aggregates – in response to the very diverse conditions confronting a particular biofilm community. This impedes efforts to control them and increases microbial tolerance to, for example, antibiotics, disinfectants and other antimicrobials. Assembly and dynamics of this intricate, active and responsive structure is mostly coordinated by secondary messengers such as cyclic-di-GMP, signaling molecules, or small RNAs, depending on the species involved. Fully deciphering how bacteria provide structure to the matrix, and thus facilitate and benefit from extracellular reactions, remains the challenge for future biofilm research.

**1. Introduction**

Biofilms are ecosystems in which the microbial cells are embedded in a matrix of extracellular polymeric substances (EPS). This matrix represents a structured and shared space, controlled by the biofilm organisms. The composition, properties and dynamics of the matrix influence the biofilm mode of life. EPS is the standard term in biofilm research1, but “extracellular matrix” (ECM) is sometimes used as an alternative2. However, the latter term includes mainly non-microbial matrices3, causing confusion when discussing biofilms in tissue culture or clinical specimens. Most recently, the term “matrixome” has been suggested, defining the entire inventory of currently known biomolecules, and their molecular, structural, and functional diversity4.

The matrix is created by the biofilm organisms, surrounding and immobilizing the cells. It comprises water-soluble polysaccharides, proteins, extracellular DNA (eDNA), and water-insoluble compounds, e.g., cellulose5, amyloids6 and non-amyloid proteinaceous fibres7 as well as lipids. Bacterially derived refractory, humic-like compounds are also reported8.

The matrix can contain multifunctional membrane vesicles, shed by the biofilm cells9,10. It can sequester material from external environments, thus, acquiring components such as dissolved nutrients, plant derived humic substances, transparent exopolymer particles (TEP)11, colloids, biotic and abiotic particles12 as well as host derived eDNA13.

The EPS-based matrix provides the infrastructure for an extremely flexible, versatile, and adaptable form of multicellular microbial life demonstrating emergent properties not shared by single planktonic or sessile cells. As early as in 1987, Costerton et al. likened biofilms to multicellular tissues14. Later, biofilms were classified as collective biological systems, such as forests, bee hives or coral reefs12. Thus, we are looking at a kind of extended organisms15, or of multicellular organisms, acting as incubators for diversity16 with a clear division of labor, e.g., in *Bacillus subtilis*2. The situation is highly complex: biofilm organisms produce diverse EPS components at various points in time during their growth, which can be influenced by other microbial species present in the biofilm. This evolution of the matrix takes place in response to the amount and nature of nutrients available and environmental conditions such hydrodynamic shear stress, pressure, salt content, temperature, light regime and grazers. In fact, the matrix exists in a continuum of physical states ranging from dissolved to dense gels17, which sometimes contain biogeominerals, either precipitated in or sequestered by the matrix, e.g., manganese oxides or calcium carbonate18.

The EPS matrix is heterogeneous in space and time, even in single species biofilms grown under controlled conditions in the laboratory. Much less is known about its heterogeneity in microbial communities in natural environments, manmade structures and certain medical and dental biofilm habitats. Millions of bacterial species exist most of which are uncultured and undescribed, all producing numerous EPS components. As an example, several million types of exopolysaccharides can be expected19. The physico-chemical characterization of various individual EPS-components in mixed species biofilms has made great progress. Nevertheless, it is still quite a challenge to identify which member in a given biofilm community produces certain components and for what function and when. In addition, current methods of analysis until recently have revealed snapshots of the matrix only, not allowing the follow up of temporal dynamics and interactions of different community members and their individual matrix compounds. For example, new methods such as the combination of Catalyzed Reporter Deposition Fluorescence-in-situ-hybridization (CARD-FISH) with fluorescent lectin binding analysis allowed to identify glycoconjugates and their 3-D localization in relation to microorganisms producing them20. More recently, stable isotope tracing combined with two-dimensional correlation spectroscopy allowed the assessment of carbon and nitrogen turnover in relation to matrix polysaccharides and matrix proteins21. Furthermore, optotracing, a method under development, was employed for selective fluorescence-based monitoring of curli and cellulose in *Salmonella* biofilms22. However, what triggers or inhibits the production of individual EPS molecules, to which extent and how it can be engineered remains largely unexplored.

Against this backdrop, it is not surprising that EPS have been termed “the dark matter of biofilms”, representing an “intractable component”8, and consequently an “identity crisis” was declared23. There is no such thing as “*the*” matrix. Very important and underappreciated are the interactions among EPS molecules and the cells surrounded by them.

In this review, we revisit the enormously growing interest and progress in analyzing and understanding the matrix of microbial biofilms as compared to our understanding more than a decade ago24. We focus on the diversity of the EPS components and novel aspects of mechanisms and consequences of their functional interactions which are central to understanding their resilience and their evolutionary success in microbial biofilms.

Furthermore, we acknowledge that the contribution of eukaryotic microorganisms to EPS composition, functions and dynamics in biofilms can be significant. Microalgae produce large amounts of matrix compounds in aquatic environments25. In addition, fungi are known to produce EPS compounds in medically important (e.g., *Candida*) biofilms, but are also considered relevant in environmental biofilms26,27. Beyond the scope of this review are the manifold ecological, technical and medical aspects of the biofilm matrix, which will be addressed in a follow-up review.

**2 The Matrix**

Much information on chemical interrogation of components in the matrix depends on the isolation method used, however, there is no universal procedure. Carbohydrates, proteins and nucleic acids are consistently found with various methods when different physical and chemical isolation procedures are compared (see suppl. Table 1). Surprisingly, these methods have not changed throughout the last decade. An exception is the use of ionic liquids for EPS extraction (see suppl. Box 1). What really has expanded are the various methods to analyze the matrix components chemically and microscopically (see suppl. Tables 2 & 3).

The components of the biofilm matrix determine architecture, function, mechanical stability and dynamics of the EPS matrix in various ways. This is the result of different and dynamic interactions of a multitude of macromolecules produced by cells as the biofilm develops. Using confocal laser scanning microscopy coefficients of molecules ranging from 10,000 to 240,000 Daltons were determined and found that they were linearly proportional to the cube roots of the molecular weights and markedly lower than diffusion coefficients in bulk water, regardless of biofilm thickness28. Interestingly, pores in the matrix can be generated by cells actively moving through the matrix (“swimmers”). These pores remain sufficiently long open to facilitate the diffusion of macromolecules throughout an established biofilm29,30.

Optotracing was employed for selective fluorescence-based monitoring of curli and cellulose in *Salmonella* biofilms22. Many EPS components can now be imaged nondestructively using specific fluorescent dyes and probes. An example is presented for a river biofilm indicating the location and distribution of lectin-specific glycoconjugates as well as polysaccharides having a β 1-3 or β 1-4 linkage such as cellulose. By means of such combinations the complex, coral reef-like structure of environmental biofilms can be generated **(Fig. 1)**.

**2.1 Polysaccharides as structural matrix elements**

For most of the history of biofilm research, polysaccharides were thought to be the major and most important matrix component, originally likened to the glycocalyx of eukaryotes31. Purification, separation, quantification and identification of polysaccharide constituents are not trivial tasks. Polysaccharides are highly diverse, not only in their monosaccharide constituents but also in glycosidic linkages, branching, and substitution with non-carbohydrate residues and charges (e.g., acetate, pyruvate, succinate, phosphate etc.), resulting in a nearly unlimited range of structures32,33. Different microbial species produce different polysaccharides, and even single strains produce multiple polysaccharides at different stages of biofilm development and depending on changing environmental conditions. For example, *P. aeruginosa* has the capacity to produce at least three polysaccharides, Psl, Pel and alginate. Polysaccharides have distinct functions in adhesion and microcolony formation and structure/architecture development of monospecies as well as in defined mixed species biofilms. Thereby polysaccharide composition significantly contributes to the emergent properties of such communities as, for example, collective protection from environmental stresses such as desiccation, immune effectors, or predators34,35. The serine protease inhibitor ecotin binds to Psl and, thus, protects *P. aeruginosa* from neutrophil elastase-mediated killing36.

Post-excretion modifications of matrix polysaccharides are well known, e.g., in length, addition or removal of substituents. Pel and poly-*N*-acetylglucosamin are partially N-acetylated, generating a heterogeneous backbone that, depending on the organism, can influence synthesis, translocation, and adhesiveness37. In *R. leguminosum*, a glycanase determines the length of the acidic polysaccharide and strongly influences the assembly and stability of the biofilm matrix. The surface-associated cellulase CelC2 plays a key role in cellulose biosynthesis by modulating the length of the cellulose fibrils that mediate firm adhesion to root tips38.

Research on the EPS of granular sludge revealed the role of α (1-4) glucans and proteins as key polymers for granule cohesion, bridged by divalent cations as major aggregative mechanism39. Recently, hyaluronic acid and sulfated glycosaminoglycan-like (GAG) polymers were found in aerobic granular sludge40. They seem to be widespread, contributing to the stability of biofilm systems, although it remains unclear whether they are formed within the granules or are imported from wastewater. Glycosylation of matrix proteins is now recognized as a way to modify functionality. An example is sialic acid which has been found in the EPS of seawater-adapted aerobic granular sludge, but also in pathogenic bacteria; this indicates the presence of sialoglycoproteins in the matrix. Sialic acid seems to be involved in the protection of sugar residues of glycoproteins against glycosidases41. Glycoproteins are suggested to be strongly involved in the structure of anaerobic ammonium oxidizing (“anammox”) granules, having a similar role as glycans in the ECM of eukaryotic organisms, e.g., in vertebrates, where they are an important constituent of the connective tissue40,42.

**2.1.1 Cellulose – an insoluble matrix polysaccharide**

Water-insoluble polysaccharides, in particular cellulose, have been identified as fundamental matrix components, influencing architecture and morphology of microbial biofilms. Although bacterial cellulose was first described by Brown as long ago as 1886 (cit. by5), it has only recently been acknowledged to be of geochemical, agricultural, biotechnological and medical importance, providing both structure and protection to biofilm bacteria34,43. Cellulose consists of polysaccharide chains of glucose units with (1🡪4) ß-glycosidic bonds. The backbone of the ß-glycosidic bonds imparts high crystallinity and considerable rigidity on the cellulose molecule, provided by the hydrogen-bonding network present among hydroxyl groups of glucosyl residues33. These hold the chains firmly together and result in fibrils with a mechanical stress resistance comparable to steel44. Cellulose plays a central role in cellular aggregation of *E. coli, Salmonella, Pseudomonas* spp*., Vibrio cholerae, Bacillus subtilis* and others in liquid cultures5. In *Aliivibrio fischeri* biofilms, cellulose affects both morphology and elasticity of the matrix45.

The ubiquity and abundance of cellulose in a wide range of microbial species led to the suggestion that cellulose is one of the most prevalent exopolysaccharides present in the biofilm matrix5. Sometimes cellulose is “decorated” by phosphoethanolamine (pEtN) substituents after synthesis46. pEtN can form nanocomposites with amyloid fibers in the EPS matrix of biofilms, resulting in tissue-like cohesion and elasticity. Further non-carbohydrate substituents of cellulose, e.g., acetyl groups, significantly affect matrix structure and stability47. As a consequence, macrocolonies are caused to give way under stress and fold into macroscopic morphological patterns of agar-grown colonies termed “wrinkled”, “rugose”, or “rdar” (for red, dry and rough)5.

**2.2 Proteins**

**2.2.1 Enzymes**

One of the most obvious functions of proteins in the matrix is that of an enzyme catalyzing biochemical reactions. Their global relevance cannot be overestimated: The primary function of extracellular enzymes is the degradation of macromolecules to low molecular weight products small enough to be transported into the cells as carbon and energy sources for microbial metabolism. Comparing activity in microbial aggregates versus that in the bulk water, specific activities were usually higher in attached bacteria48. Comprehensive overviews of matrix enzymes and their functions are available49,50. As the enzymes can be retained, e.g., by polysaccharides51 or humic substances52, they are kept in close proximity to the biofilm cells, resulting in an external digestion system12.

The role of extracellular enzymes is not restricted to polymer degradation; they are important for the modulation of the matrix, particularly through polysaccharide-degrading hydrolases, esterases, proteases and lyases, which allow for continuous restructuring (see Box 3). Thus, enzymes are the carriers of the structural dynamics of the matrix by causing localized modifications with concomitant changes in the chemical and physical properties such as gelation behavior, ion binding and mechanical stability24. Extracellular enzymes can act as virulence factors; an example is the massive interference of proteases with wound healing after infection53. Enzymes are crucial for the detachment and dispersal of biofilms54,55. Beyond their function as enzymes, proteins provide important functional and structural features of the biofilm matrix.

In natural ecosystems, the turnover of biopolymers represents the rate-limiting step in the mineralization and cycling of particulate and dissolved organic matter. This process is performed by extracellular enzymes. The majority of microorganisms on Earth live in biofilms56, driving all biogeochemical cycles57. Their extracellular enzymes transform enormous quantities of dissolved polymers globally and render particulate substrates bioavailable, thus, these enzymes represent the bottleneck of the planetary self-purification processes58,59.

**2.2 2. The ubiquitous functional amyloids**

Amyloids are water-insoluble highly ordered proteins characterized by a strongly conserved quaternary structure, i.e., a fibrillar morphology 7–13 nm in diameter, and a β-sheet secondary structure. This is shared by all amyloids60. They can be stained by dyes such as Congo red, Thioflavin T and CDy11. The term amyloid entails a biophysical, and not a biological, description. The morphology and biophysical characteristics of all amyloid fibrils are remarkably similar, although the primary structure of amyloid proteins may differ strongly61. Strikingly, amyloids are self-assembling in a nucleation-dependent process from their monomeric constituents given the right environmental conditions62. In contrary to amyloids as misfolded plasma proteins associated with brain diseases in humans, amyloids in biofilms fulfil manifold biological functions. They show a structural, biochemical and functional versatility that microbes exploit in different contexts and for different purposes63, including the enhancement of virulence64.

They represent an important and frequent structural component of biofilm matrices6, that help to maintain homeostasis15. They can stabilize the biofilm matrix or be involved in cell division, nutrient storage, surface-tension modulation, cell cycle regulation, cell-cell communication, adhesion, flocculation, desiccation resistance, cytotoxicity, virulence and evasion of the host immune system65. Furthermore, they contribute to resistance towards mechanical, thermal and chemical stressors of biofilms6. Amyloids such as curli in *E. coli*6,66, functional amyloids in *Pseudomonas*67, the extracellular protein TasA68, biofilm-associated proteins (baps)69 and phenol-soluble modulins (PSMs)70 have all been comprehensively described. Some methanogens even use amyloids as a sheath attributing to cell stability and stiffness71.

**2.2.3 Hydrophobic regions in a hydrophilic matrix**

Although highly hydrated, the matrix can still contain hydrophobic regions. Hydrophobic matrix proteins (hydrophobins) have been studied mostly in *B. subtilis.* The biofilm surface layer protein A (BslA) secreted by *B. subtilis* can form a hydrophobic film coating the biofilm surface, rendering it water-repellent72,73. This molecule undergoes an environmentally responsive conformational change upon partitioning from an aqueous to a hydrophobic environment, e.g., a hydrophobic surface or the air-water interface, and has been termed a “hydrophobic raincoat”73,74. Once at an air-liquid interface, BslA self-assembles into an ordered rectangular 2-D paracristalline lattice that forms a macroscopically visible elastic protein layer75. Three categories of BslA and its variants based upon their wrinkle relaxation behavior were proposed72: I) surfactant-like, II) transiently wrinkling biofilm formers, and III) rigid biofilm formers. It was suggested that the hydrophobic surface of a biofilm helps to prevent erosion of cells and, at the same time, retain humidity in the underlying matrix and thereby protects biofilm cells from water stress76.

Although the matrix is generally considered as a hydrophilic environment, activated sludge flocs from wastewater treatment plants can accumulate hydrophobic pollutants77. It was shown that they were associated with the matrix and not the cells, clearly indicating hydrophobic domains24,78. Protein interactions can form hydrophobic pockets with amyloids79; for example, BslA self-assembles to an elastic hydrophobic film, coating the air-, solid- and liquid cell interface of *B. subtilis* biofilms75. In *Shewanella oneidensis* biofilms, such areas were demonstrated80, using amphiphilic surface-engineered quantum dots. Proteins such as Bap1 and BslA have been described as hydrophobic72,73,76. Not only proteins but also single-stranded eDNA can increase the hydrophobicity of cells81. Nevertheless, a mechanistic understanding of hydrophobic domains in the biofilm matrix is still lacking.

**2.3 The manifold functions of extracellular DNA (eDNA)**

eDNA has been identified as an important feature of the biofilm matrix, representing much more than merely an inert structural element82. It is one of the most frequently found matrix polymers81. Different mechanisms are involved in eDNA release, such as autolysis and active secretion as well as release via membrane vesicles. The origin of eDNA appears to be genomic DNA (gDNA)81 and mostly lysis-related83. However, eDNA is not always structurally or compositionally identical to gDNA84. In several bacteria, there is a relationship between eDNA release and bacterial competence to take up DNA from the environment83.

The biological functions of eDNA include roles in adhesion and primary stages of biofilm development, as a scaffold component, in gene transfer and DNA damage repair, and as a source of organic carbon, nitrogen and phosphate85. G-quadruplex eDNA structures, as analyzed after ionic liquid extraction (see Box 1), build viscoelastic networks in *P. aeruginosa*, contributing to matrix stability86. One function of eDNA is that of a potential genetic pool for some bacteria which can take up and recombine with homologous DNA fragments by natural transformation87.

DNA interactions with other biomolecules on the cell surface and in the EPS matrix seem to be mediated by unspecific electrostatic interactions, aided by divalent cations81. eDNA was found to bind to proteins of the DNABII family in single- and mixed-species biofilms. These molecules stabilize the eDNA scaffold and are localized at the vertices of the eDNA lattice structure and have a high affinity for branched DNA structures which include Holliday junctions (HJ)88. HJs are single-stranded cross-over intermediates of homologous recombination. DNAse resistance in mature biofilms has been suggested to be due to the formation of left-handed Z-DNA; this molecule seems to serve an important structural role in biofilms by interacting with DNAB II proteins89.

Some DNA-amyloid complexes play a role in the modulation of the mechanical resistance of *B. licheniformis* biofilms90. Through electrostatic binding of drugs, eDNA provides a shielding function against charged antibiotics and cationic antimicrobial peptides82. It further contributes to antibiotic tolerance by directly inactivating cationic antibiotics91. In *Clostridioides difficile,* eDNA is essential for biofilm formation and structural integrity92. *S. aureus* biofilms have been reported to be stabilized by positively charged cytoplasmic proteins which are released into the extracellular environment, where they interact electrostatically with the negatively charged cell wall and eDNA93. eDNA binding lipoproteins contribute to *S. aureus* biofilm formation by linking bacterial cells through cross-links with eDNA. With the biofilm maturing, eDNA enters a nuclease-recalcitrant state, likely to further protect the resident bacteria in the biofilm94. e-RNA was also found in the matrix, associated with outer membrane vesicles of *E. coli*95.

**2.4 Membrane vesicles**

Membrane vesicles (MVs) are produced by all domains of life. They consist of lipid bilayers forming spheres 20 – 500 nm in diameter and are released by bacteria, archaea and eukaryotic microorganisms96. Bacteria produce physiologically heterogeneous types of MVs97, and the same species can release different types of MVs98. Outer membrane vesicles (OMVs) derived from the outer membrane of Gram-negative bacteria have been found to be ubiquitous in the matrices of bacterial biofilms including laboratory biofilms like those of *P. aeruginosa,* as well as biofilms in natural and technical aqueous environments99. OMVs can be considered as “nano-parcels” travelling across the matrix, containing cytoplasmic material100, e.g., nucleic acids, toxins, lipoproteins and enzymes101, effector proteins, DNA102, antibiotics10 and signaling molecules103, lipids, adhesins, DNA, ATP, toxins, virulence modulating factors104, ATP105, or hydrophobic signaling molecules106. OMVs of Gram-negative bacteria are involved in bacterial survival under stress and regulate the interactions among members of Gram-negative bacterial communities104. In an oxacillin-sensitive methicilline resistant *Staphylococcus aureus* (MRSA) strain, antibiotic stress (exposure to ceftazidime) induced biofilm formation with the production of membrane vesicles as structural components with increased hydrophobicity, mediating intercellular junctions in the biofilms107. Release of OMVs from cells enhances biofilm formation of *Aeromonas* strains108. OMVs have been described as instrumental for stepwise cellulose hydrolysis109. Gram-positive cells also can shed membrane vesicles101. Phage-encoded endolysin generated holes in the cell walls of *Bacillus subtilis* through which cytoplasmic membrane material protruded into the extracellular space, released as MVs110. This induced OMV formation in neighboring cells by the action of the released endolysin.

**2.5 Humic substances and recalcitrant compounds**

Humic substances are known as components of the soil organic matter that have non-specifically auto-assembled in supramolecular associations and are composed of a vast variety of macromolecules111. It is debatable whether humic substances should be included in the definition of EPS and whether they can be modified within the matrix. In this review, we consider them common constituents present in the matrix under environmental conditions, e.g., water and soil, when biofilms come into contact with these exogenous compounds, which may also participate in matrix processes. For this to occur several topics must be considered: I) the microbial carbon pump in the ocean112 and in soil113; II) dissolved and particulate organic matter (DOM/POM)112 and soil organic matter (SOM)113; and III) bacterially derived refractory compounds and necromass114. Key findings suggest that microorganisms are responsible for the production of long-term stable constituents and particulates, all of which will contribute to recalcitrant compounds and microbially derived humic substances present in the biofilm matrix. The remains of lipid containing compounds such as bacterial cell envelopes may be potential candidates for hydrophobic domains within the biofilm matrix115, together with rhamnolipids116. This would help to understand the finding of hydrophobic domains as measured with amphiphilic Q-dots in combination with confocal laser scanning microscopy80.

**3. How and why do matrix components interact**

Structure, functions, mechanical stability and dynamics of the matrix are based on various interactions between a multitude of macromolecules and metabolites produced by the cells during their life cycle in the biofilm. The current understanding of the matrix goes beyond identification of individual components and their molecular interactions. Consequently, one has to consider both biological and ecological functions of these interactions as well: The matrix is not simply a passive scaffold and a protective space but can actively and reactively influence the cells in the biofilm and, thus, the dynamics of biofilm development (see supplementary Table 4). Functional interactions turn the matrix into a feedback system with the biofilm117, regulating proliferation, intra-biofilm migration and differentiation, thereby interacting with biofilm cells and influencing their physiological characteristics. It is these functional interactions that generate many of the emergent properties of biofilms.

The matrix is ideally suited to these functions. It represents a confined space in which the inherently high concentration of EPS molecules strongly promotes all kinds of physico-chemical interactions and can be likened to molecular crowding inside microbial cells118. It results in the viscoelastic, but mechanically stable and highly adaptable structure which develops during biofilm formation. Not all interactions are cohesive; steric repulsion is another aspect of macromolecular crowding119, preventing the collapse of the matrix. Therefore, the physico-chemical molecular interactions are pivotal to the biofilm mode of life. From a molecular point of view, the interactions between EPS components are dominated by weak physico-chemical interactions such as hydrogen bonds, hydrophobic, electrostatic and ionic interactions, or cross-linking by multivalent cations between EPS molecules and between cells24. It is the high number of monomeric constituents and, particularly in the case of polysaccharides, the presence of substituents capable of binding, which multiply the weak forces of single bonds to considerable overall cohesion, even if only a small fraction of them connects. Their reversible, rapid association and dissociation provide their transient nature, and, thus, the flexibility of the matrix. A further important factor contributing to matrix stability is the physical entanglement of EPS strands8,120.

A comprehensive view of the temporal and spatial dynamics of biofilms is difficult to obtain. One of the few successful examples is the development of *Vibrio cholerae* biofilms. Multiresolution imaging revealed the complementary architectural role of four essential matrix components: the protein RbmA mediates cell-cell adhesion, the protein Bap1 provides adhesion of developing biofilms to surfaces, and the combination of *Vibrio* polysaccharide (VP), RbmC and Bap1 forms dynamic, flexible and structured envelopes that integrate biofilm cell clusters121,122.

The interactions of EPS components fulfil many crucial functions. An example is the retention and stabilization of degradative enzymes (e.g., lipase) by bacterial polysaccharides (e.g., alginate), preventing their loss into the environment. eDNA can interact with polysaccharides123,124 and proteins, modulating and strengthening the matrix93,123. In *P. aeruginosa* biofilms the crosslinking of the exopolysaccharide Pel125 with eDNA provides protection against enzymatic attack from DNase13. Of particular interest is the function of the exopolysaccharide Psl as an intercellular signaling molecule that actively stimulates cellular c-di-GMP production, thus amplifying the biosynthesis of their own matrix in a positive feedback circuit117,126. EPS molecules can retain iron ions which in turn promote biofilm formation by downregulating the synthesis of rhamnolipids, which in turn elevates Psl production in *P. aeruginosa*127. In *P. aeruginosa,* amyloids in the matrix can temporarily store in and accumulate the signaling molecules including N-acyl-L-homoserine lactones (AHLs) and *Pseudomonas* quinolone signal (PQS)128. In mucoid *P. aeruginosa*, overproduction of the exopolysaccharide alginate interferes with the response to *Pseudomonas* quinolone signal (PQS), but not to AHLs117. In addition to its mechanical role, eDNA has been shown to facilitate the production of amyloid fibers in the EPS matrix of *S. aureus* biofilms by promoting the polymerization of phenol soluble modulins (PSM)79. To add further complexity, the same study found that amyloid fibers induced autolysis and the release of eDNA, in what we interpret as a positive feedback loop of EPS production79, not dissimilar to that of the *P. aeruginosa* polysaccharide Psl126, albeit via a different mechanism. These examples indicate that EPS components can modulate cell-cell communication, and a new function was ascribed to EPS: the ability to function as signaling molecules that the cells can receive and respond to117,126. Outer membrane vesicles129 and proteins88 have been shown to bind to eDNA to increase cellular aggregation and biofilm structural stability and protect eDNA from degradation. Phenazine is a redox-active metabolite providing *P. aeruginosa* the green color. It has significant roles in signaling, biofilm development, iron-acquisition, cell metabolism, and antibiotic tolerance130. In *P. aeruginosa* biofilms, phenazine131 as well as pyocyanine132 interact with eDNA resulting in enhanced electron transfer among biofilm components, better eDNA binding to cell surfaces and increased biofilm viscosity131. Phenazine as well as pyocyanine132 interact with eDNA resulting in enhanced electron transfer among biofilm components132. In *P. aeruginosa* biofilms, phenazine as well as pyocyanine132 interact with eDNA resulting in enhanced electron transfer among biofilm components132. Furthermore, Mg2+-ions sequestered by eDNA trigger the expression of resistance genes in *P. aeruginosa*81.

Thus, the matrix can be considered a continuously interactive, self-regulating, shared space - not just a passive structure and protective material of biofilms. Such functions are made possible by manifold interactions of the matrix components (**Figure 2**). There is increasing evidence that EPS components can be versatile in their multiple and sometimes redundant biological functions based on interactions with diverse ions, signaling molecules and biopolymers in response to the prevailing conditions of biofilm composition and development.

**4. Regulation of EPS matrix synthesis**

Matrix formation, maintenance and transformation are highly regulated by multiple, species-specific systems through both genetic control on the cellular level and environmental factors. Most of the information has been gained from single species in laboratory settings, already demonstrating a wide diversity of strategies; multiple-species environmental communities will be even more complex. Certainly, there is no single regulatory mechanism for all EPS components in all biofilms at all development stages and environmental situations, but there is a huge variety of regulation pathways and networks with which different microorganisms are genetically equipped and in which they can respond to environmental factors. However, some common principles can be identified and are shown in **Figure 3** in a simplified schematic.

Different species manage their regulation in different ways while extrapolations of single strain studies to mixed species biofilms appear still problematic. Consequently, it is not yet feasible to manage biofilms in technical systems with the aim to over- or under-produce EPS, with the possible exception of biogranulation133. Abiotic factors such as nutrient levels, temperature, osmolarity or shear forces will also influence regulation. Furthermore, biofilms are rarely homogenous but characterized by heterogeneous cellular physiology and architecture, because of gradients of nutrients and terminal electron acceptors, like oxygen, that are further accentuated during biofilm growth. A consequence is the heterogeneous production of matrix components. In general, a fully developed biofilm is not only the result of a specific genetic programme of developmental differentiation, but also of growth and transport limitation134, all of which lead to a spatially differentiated multicellular environment.

Nevertheless, the control of matrix components by intra- and extracellular signals is achieved I) transcriptionally135, II) post-transcriptionally126, and III) by environmental factors136. Commonly, the control pathway starts with sensors, followed by signal transmitters and ending with effectors which, in turn, execute production or modulation of factors involved in biofilm formation, maintenance and dispersion/detachment135. In general, quorum sensing (QS) signaling molecules called autoinducers, the intracellular second messenger cyclic-di-guanosine-5´-monophosphate (c-di-GMP) and small RNAs are considered main bacterial regulators as transmitters in the signal transduction pathway, but in very complex ways, with considerable variations in QS molecules and sRNAs. Four examples are briefly presented here:

***P. aeruginosa.*** Biofilm formation is regulated by three known systems135: I) QS molecules and chinolon systems which sense cell density and activate LasI and RhII with their effectors LasR and RhIR. This results in the synthesis of rhamnolipids and the secretion of eDNA. II) c-di-GMP sensed by receptors such as WspA. The signal is transmitted by diguanylate cyclases and phophodiesterases to effectors and repressors such as Alg44 initiating the synthesis of adhesins and exopolysaccharides and inhibiting motility. III) Small non-coding RNA molecules, involved in a regulation pathway viasensor kinases such as GacS, phosphorylation of GacA, which activates transcription of the small RNAs *rsmY* and *rsmZ* which reduce the activity of the effector protein RsmA resulting in inhibition of Psl exopolysaccharide synthesis and enhancement of motility135, 137. In *P. aeruginosa*, LasB could promote biofilm formation partly through rhamnolipid-mediated regulation127. The functional diversity of polysaccharides at different points in time is orchestrated by complex regulatory mechanisms ensuring that the correct polysaccharide is produced at the right time and location within the biofilm35.

An additional signaling function was discovered for the matrix exopolysaccharide Psl126. It stimulates the production of c-di-GMP whose elevated intracellular concentrations then lead to the increased production of Psl. Recently, the *Bacillus subtilis* exopolysaccharide was also identified as a signaling molecule that stimulates its own production mediated by a membrane-associated tyrosine kinase138. These self-regulation mechanisms are based on a positive feedback regulatory loop linking production of the exopolysaccharides to their concentration in the biofilm. These results indicate the potential of matrix exopolysaccharides to act as short-range signaling molecules and function as a common good in the biofilm community126,138.

***V. cholerae.*** The formation of biofilms and EPS in *V. cholerae* is well understood121,139.The *Vibrio* polysaccharide (VPS), main constituent of the matrix, is encoded by 12 genes regulating the enzymes involved in producing the nucleotide sugar precursors, glycosyltransferases, polymerization and export proteins, acetyltransferases, and phosphotyrosine-protein phosphatase. The matrix proteins RbmA, RbmC, and Bap1 are produced and secreted at various times during biofilm formation. The type II secretion system, composed of multiple export proteins, is responsible for the secretion of RbmA, RbmC and Bap1. In aquatic environments, salinity and osmolarity fluctuations as well as phosphate limitation can affect VPS gene expression and biofilm formation. As a key signaling molecule, c-di-GMP controls motility and biofilm formation of *V. cholera.* Similarly, small RNAs have a role in gene regulation15.

***E. coli.*** In elegant work it was shown that in *E. coli* macrocolony biofilms, a matrix of proteins, amyloid fibres, exopolysaccharides such as phosphoethanolamine (pEtN)- cellulose and eDNA form a robust matrix with pronounced stress resistance and spatial, physiological differentiation in the supracellular architecture5,15. Gradients of nutrients, oxygen, waste products and signaling molecules determine whether the cells grow and proliferate or enter stationary phase according to microscale spatial conditions. The heterogeneity of the matrix influences the architecture, physical properties, and morphology of the macrocolonies. The extracellular matrix components pEtN-cellulose and amyloid curli fibers are major targets of the underlying control network. C-di-GMP specifically controls pEtN-cellulose production allowing the cells to alter the ratio of pEtN-cellulose to amyloid curli fibers and thus to adjust the mechanical properties of the matrix in different zones of the biofilms5,15.

***S. aureus.*** In *S. aureus,* biofilm development and virulence are regulated through a number of two-component systems. Genes in the accessory gene regulator (*agr*) system140, which regulates the production of proteases and phenol soluble modulins (PSMs), involved in biofilm assembly and disassembly, are best described. Interestingly, the expression of secreted virulence factors is inversely related to biofilm formation. In *S. aureus,* the EPS are initially dominated by eDNA, which is released through autolysis. Cells can adhere to the eDNA through DNA binding lipoproteins, providing stability93. In this early stage, bacterial cells can be released from the biofilm by the production of nucleases in what has been termed the “exodus” stage141. In the early stages of development, PSMs reduce biofilm formation through their surfactant effect. PSMs can also play a direct positive role in EPS production through their polymerization into amyloid protein fibrils, and in this later developmental stage the EPS matrix is increasingly proteinaceous140. Active release of cells from the biofilm at this stage is achieved through the production of proteases. Complex structural and regulatory interactions between EPS components in *S. aureus* are likely. The presence of eDNA has been shown to promote the formation of amyloid fibers by phenol-soluble peptides79. To add further complexity, the same study found that amyloid fibers induced autolysis and increased the release of eDNA, in what appears to be a positive feedback loop. The regulation of multiple-species biofilms and how biofilm organisms influence each other spatiotemporally remain important questions in biofilm research.

**5. The house of biofilm cells and its architecture**

The architecture of microbial biofilms is determined by several factors. The major drivers are the hydrodynamics, substratum, temperature, nutrient limitation and ratios of available C, N, and P, as well as trophic grazing, and susceptibility to bacteriophages142. The observer’s perception of a given biofilm is influenced by the microscopic technique applied. In fact, the real architecture is only revealed by visualizing the fully hydrated biofilm. For this purpose, laser scanning microscopy (confocal/one-photon and multi-photon) evolved as a key analytical technique143. More recently, optical coherence tomography (OCT) and selective plane illumination microscopy (SPIM) have emerged as useful techniques144,145. Since the first substantial report146, numerous publications of biofilm micrographs show the architecture of laboratory-grown single- and multiple-species biofilms, as well as biofilms found in the natural and built environment. Taken together, they illustrate how variable and diverse biofilms are, but delineating their features microscopically comes down to the visualization technique applied, the magnification and the way the undisturbed biofilm is prepared and mounted. However, the cartoons of biofilms derived from such images are usually bordered by a line, defining the inner space of the biofilm. This is questionable. In reality, such a strict biofilm edge does not exist but rather there is a more or less steep gradient in EPS concentration, and mostly, the biofilm is ragged at its periphery at the surrounding water phase. In some cases, exposed microbes at the biofilm-water interface don´t even seem to show a matrix around them.

Three examples of environmental biofilms give some insight into the architecture of the matrix microenvironment. In most biofilm systems water and EPS constituents are not homogenously distributed within the matrix as there are pores and channels as well as clusters of matrix polymers147,148. Three zones of EPS form a common pattern: cell-associated, inter-cellular and an outer layer of EPS as imaged by lectin binding analysis. This arrangement provides a scaffold, which can segregate extracellular activities at the microscale as well as differentially binding contaminants. Thus, the biofilm matrix as highly hydrated structure will show certain spatial features, especially in thick gel-like biofilms. For example, methanol fed biofilms developed in rotating annular reactors grew up to 4 mm thickness concomitant with an extensive production of a nearly transparent matrix149. The matrix examined with a range of probes showed a globular architecture with bacteria in between as well as individual cells in the center of the globules. In a different habitat, matrix-dominated biofilm structures found in semi-artificial caves show new structural matrix features not seen before **(Figure 4)**. The massive biofilms covered the walls and ceilings with pendulous mucous structures, termed “snottites”, up to 15 cm in length150. Imaging of cells and EPS by means of CLSM in combination with fluorescent lectin-binding analysis151 revealed extended capsular features often linked to globular strands with voids, but also multilayered spherical globules with higher levels of organization. The globular matrix structures, depending on their biochemical composition, may influence the flux of low and high molecular weight compounds in and out the biofilm. In addition, thin wire-like glycoconjugate strands seem to stabilize the gelatinous structure of snottites.

Molecular techniques such as genomics, transcriptomics and proteomics focused in part on the extracellular space. So far, several –omics techniques applied to biofilm microbes and biofilm matrices delineated by Neu & Lawrence unraveled a number of proteinaceous constituents with new and unknown functions143,152. A notable approach missing to complete the -omics is glycomics in order to address the different polysaccharide constituents of the matrix. In terms of new developments in matrix visualization, image scanning microscopy with double resolution if compared to confocal microscopy, may supplant traditional laser microscopy techniques. Furthermore, laser-based “nanoscopy” applied to fully hydrated biofilms has great potential to provide information on the dynamics and spatial interactions between EPS components and microbial cells153.

**6. Stability of the scaffold: mechanical properties of the EPS matrix**

Viscoelasticity is a corollary of the self-assembly of matrix components which create the structure of biofilms154,155. Biofilm mechanics is an important field providing clues about how biofilms respond and adapt to physical forces. This is highly relevant for attached biofilms exposed to shear stress and free-floating aggregates prone to collisions. Adaptability in mechanical response is of particular importance for the survival in environments where physical forces can fluctuate greatly156.

Mechanically, biofilms are generally considered as highly hydrated hydrogels157. Their macroscopic rheological properties are determined at the molecular level, e.g., by electrostatic interactions and entanglement between the EPS polymers and charged molecules or ions. More recently, complex eDNA network structures including those mediated by extracellular nucleotide binding proteins88,158 have been found to also have a mechanical function. Although the microbial cells produce the matrix polymers, in conceptual models explaining mechanical properties they are not considered relevant.

A key feature of the reported mechanical properties of biofilms is their viscoelastic nature, even though the degree of elasticity (how much the biofilm stretches and springs back) and viscosity (how much the biofilm flows) under mechanical stress vary over many orders of magnitude, even for single-species biofilms159. There are two main reasons that explain such variability. The first, and biologically most interesting, is that biofilms respond to different nutrient and hydrodynamic conditions structurally and mechanically over very short time spans (milli-seconds)160, or adapt gradually (days to weeks)161. The rheological behavior of biofilms can range from resembling elastic solids162 to low viscosity liquids163 **(Figure 5)**, providing an enormous range of survival functionality. This incredible versatility is achieved by the weak and non-specific physico-chemical interactions of the macromolecules. The concept of “fluctuating binding points” was suggested to explain the viscoelastic state of the matrix and the rapid transition from elastic solids to highly viscous fluids depending on the magnitude of applied mechanical stresses24,164. These interactions occur between polysaccharides, proteins, eDNA and all other biopolymers in the matrix and can be controlled by the water content through regulation of polymer density165. It makes sense in terms of adaptive response that covalent bonds between the EPS components have not been reported since such bonds would make the matrix more rigid, less dynamic and less capable to rapidly respond to changing physiological and environmental conditions. Multivalent cations added to the surrounding liquid or even cationic polymers produced by the bacteria themselves can cause cross-linking of negatively charged polymers, such as alginate and eDNA, within the biofilm causing biofilms to become more solid-like (i.e. less compliant)166. However, it is not clear whether the degree of such ionic cross-links is controlled by the biofilm bacteria or happens incidentally due to the chemistry of the local environment.

The second reason for variability is that biofilms are non-linear materials167, and the measured mechanical properties are highly dependent on the test methods168. At first glance testing appears a relatively simple process of applying a mechanical force and measuring a physical change in length, shape or volume, or *vice versa*. However, how sample preparation is done and whether the testing is done under shear, compression or tension, as well as the rates, duration and magnitudes of the applied perturbations, and the scale of the testing, will influence the outcome.

The testing method should relate to the forces the biofilm may experience in its natural environment. The mechanical properties of biofilms can be considered multi-scalar ranging from single cell adhesive interactions to the bulk properties of whole biofilms treated as a continuum material169. Even if the biofilm bacteria are killed, the material properties of the matrix may nevertheless remain unchanged170. The matrix can be shared by other organisms which did not contribute to its synthesis and assembly. However, in *B. subtilis* it was shown that the matrix remained partially controlled by the producer subpopulation where cells stick together even under shear stress67. Higher levels of structural order emerge as whole biofilms form bridging networks171, which undoubtedly will also influence the mechanical properties of the overall structure. There is a link between mechanical properties and antibiotic susceptibility172. It is not surprising that these are correlated since the EPS-cell-water relationships that determine the mechanical properties will also be important for determining the rates of transport of antimicrobial agents and nutrients (and therefore cellular metabolic activity) through the biofilm. Thus, while the biofilm mechanics, due to promotion of persistence of the cells can be considered a virulence and environmental survival factor in its own right154,155, it belongs to a subset of factors governed by the combined physics and chemistry of the EPS.

**7. Conclusions and outlook**

In the last decade, great progress has been made in understanding the pivotal role of the EPS matrix for the biofilm mode of life. This is reflected in a broader definition of biofilms which embraces the widely diverse phenomena of microbial aggregates, all sharing common features and the special environment provided by the EPS matrix173. The complexity of the matrix has been demonstrated; it is not just a blob of slime made of polysaccharides but provides a dynamic, functional and protective shared space for biofilm cells. The synthesis, export and assembly of matrix components is determined by multiple interconnected systems, and bacterial species may differ in terms of matrix components synthesized and regulatory pathways.

Much progress has been made in the analysis of the EPS components and the fine structure of the matrix, and a shift of focus has unfolded the paramount role of proteins. Extracellular enzymes in the matrix are the tools with which biopolymer transformations are accomplished, and amyloids are ever-present as structural, protective and functional EPS constituents. eDNA has also been recognized as having multiple and pivotal functions in the matrix. Finally, water as the largest constituent of the matrix has found appropriate attention174. Of particular interest are the functional interactions of EPS molecules, which extend beyond influencing the mechanical stability of the matrix.

Of course, there are technical, environmental and medical aspects to be addressed, as for example, the role of EPS in the tolerance of biofilm organisms to antimicrobial agents. Among the less well studied but deserving aspects ranks the effect of EPS on the stability and movement of sediments in rivers and estuaries and their ecological function as a “microbial skin” of streams and rivers58. The matrix can act as a sink and source for sorbed organic and inorganic contaminants, which can interfere with the agricultural use of sewage sludge because of such contaminations. EPS are involved in the resistance to erosion and humidity balance of soils and thus, have a direct impact on agriculture. In water and wastewater treatment, EPS allow for the retention of biofilms in biofilters, or as the glue that keeps activated and granular sludge together, allowing for separation of biomass from water. In medicine, the EPS are involved in tolerance towards antimicrobials (see Box 2). Furthermore, biotechnological aspects of EPS deserve attention. For example, their adhesion properties and mechanical stability have to be overcome in cleaning and other anti-fouling strategies, and also involved in microbially influenced corrosion. Such aspects would have exceeded the scope of this review and will be covered elsewhere.

What also remains open is how to control EPS production and composition, which would be very attractive in terms of environmental engineering, for example for carbon sequestration or bioremediation and in technical engineered systems such as waste water treatment or fuel cell development. As an example, increasing the EPS concentration in soils could improve humidity retention. Conversely, minimizing EPS production, or decreasing their adhesion strength or increasing their permeability and cleanability, could constitute an anti-fouling strategy in membrane processes.

We must bear in mind that most chemical and functional information about EPS has been generalized from a relatively small number of experimental laboratory models, often microorganisms of clinical relevance, and these bacteria are uncommon in biofilms found in nature or the built environment, like wastewater treatment plants23. The use of next generation DNA sequencing combined with other -omics tools appears as a promising way to identify signature EPS components in many environmental biofilms and may help understand their regulation. Here, *in-situ* approaches will be important. Chemical imaging becomes an increasingly helpful tool to analyze complex microbiota and connect this with EPS chemistry23.

Not surprisingly, the deeper we peer into the structure, function and regulation of components in the EPS matrix, the more of its complexity is revealed. Another exciting aspect is that the maintenance of biofilms is discernible as an active process; preliminary research on *P. aeruginosa* indicates that maintenance regulation is mainly performed by transcriptional regulation and secondary messenger signaling175. Furthermore, it is still unclear if EPS functionality is similar across different biofilm systems. After all, the matrix evolved over billions of years, enhancing the odds of survival for sessile cells living at interfaces, often under extreme environmental conditions.

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**Legends**

**Figure 1: Confocal laser scanning microscopy (CLSM) of an epilithic river biofilm.**

A) Multichannel (5) image dataset visualised as 3-dimensional volume projection. The channels presented individually show an insert symbolizing the biochemical nature of the signal. B) Volume of bacterial cell distribution. C) Autofluorescence of chlorophyll A indicating algae. D) Massive volume of lectin-specific glycoconjugates containing fucose. E) Volume of β 1→3 and β 1→4 polysaccharides such as cellulose. Please take notice that the combined image in A) diminishes part of the signals due to their 3-dimensional location and the dense biofilm structure.

Colour allocation: White = mineral reflection, green = SybrGreen nucleic acid stain, blue/purple = autofluorescence of phototrophic microorganisms, red = AAL-lectin-stained glycoconjugates, yellow = CalcofluorWhite-stained polysaccharides. Grid size = 50 µm.
(Courtesy: sample - U. Risse-Buhl, CLSM – U. Kuhlicke, visualisation – T.R. Neu)

Detailed legend for supplement

Confocal laser scanning microscopy of an epilithic river biofilm. The sample was fixed in 3.7 % formaldehyde for 2 hours, then the sample was kept in sterile river water at 4°C in the dark.

Mounting and staining: pebble in Petri dish, lectin of *Aleuria aurantia* AAL-Alexa568, SybrGreen, CalcofluorWhite M2R.

Microscopy: Leica TCS SP5X, upright microscope, software LAS AF 2.4.1., super continuum laser light source, 25x NA 0.95 water immersion lens, step size 1 µm. Sequential scan: First 490 nm (reflection, SybrGreen) and 633 nm (chlorophyll A) excitation. Second 405 nm (CFW M2R) and 578 nm (lectin) excitation.

The multichannel (5) image dataset was visualised with Imaris ver. 9.8 using Surpass / 3d view as 3-dimensional volume projection in Blend mode.

Colour allocation: White = mineral reflection, green = SybrGreen nucleic acid stain, blue/purple = autofluorescence of phototrophic microorganisms, red = AAL-lectin-stained glycoconjugates, yellow = CalcofluorWhite-stained glycoconjugates. Grid size = 50 µm.

(Courtesy: sample - U. Risse-Buhl, CLSM – U. Kuhlicke, visualisation – T.R. Neu)

Reference: Risse-Buhl U, Anlanger C, Kalla K, Neu TR, Noss C, Lorke A, Weitere M (2017) The role of hydrodynamics in shaping the composition and architecture of epilithic biofilms matured in fluvial ecosystems. Water Research 127: 211-222
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**Figure 2: Interactions of EPS components and their functions.** (1) Outer membrane vesicles transporting matrix components, (2) Crosslinking of polysaccharide chains (PS1 and PS2) by proteins such as CdrA, (3) Protection against host proteases by protease inhibitor (ecotin) associated with matrix exopolysaccharide (Psl), (4) Retention of exoenzymes, formation of extracellular digestion system, (5) Stabilization of eDNA lattice by proteins (DNABII), (6) Matrix stabilization and eDNA protection by interaction with amyloids and polysaccharides, (7) Enhanced electron transfer with eDNA, (8) Complexation of charged peptides with eDNA, (9) Matrix stabilization by eDNA-cation complexing, (10) Anchoring the matrix to biofilm cells, (11) Promotion of cell aggregation. (For details, see supplementary Table 4.)

**Figure 3: Regulation of matrix synthesis**

Simplified diagram of major factors influencing the formation and release of matrix compounds.

**Figure 4: Matrix snottites**

Confocal laser scanning microscopy (CLSM) of snottites from a semi-artificial cave. Notable are the two types of glycoconjugates showing globular and tube-like shapes (green) in combination with interconnecting thin strands (red) as well as bacteria (blue). The insert shows a macroscopic view of several snottites of about 5 cm length. The multichannel image dataset was visualised as 3-d volume projection. Colour allocation: White = mineral reflection, green = AAL-A488 lectin stain and red = PNA-TRITC lectin stain both for glycoconjugates, blue = Syto60 nucleic acid stain. Grid size = 10 μm. (Courtesy: C. Karwautz and U. Kuhlicke)

Detailed legend for supplement

Confocal laser scanning microscopy (CLSM) of snottites from a semi-artificial cave. The sample was fixed in 3.7% PFA for transport.

Mounting and staining: the snottite was cut in sections using a scalpel and placed in a 2 mm deep cover well chamber. For glycoconjugate staining the lectins *Aleuria aurantia* AAL-Alexa488 and *Arachis hypogaea* PNA-TRITC were applied, counterstaining for nucleic acids was done with Syto60.

Microscopy: Leica TCS SP5X, upright microscope, software LAS AF 2.4.1., super continuum laser light source, 63x NA 1.2 water immersion lens, step size 0.5 μm. Sequential scan: First 500 nm (reflection, AAL-Alexa488) and 633 nm (Syto60) excitation. Second 555 nm (PNA-TRITC lectin) excitation.

The multichannel (4) image dataset was visualised with Imaris ver. 9.8 using Surpass / 3d view as 3-dimensional volume projection and maximal rendering quality in MIP mode.

(Courtesy: C. Karwautz and U. Kuhlicke

Reference: Karwautz, C., Kus, G., Stöckl, M., Neu, T.R. & Lueders, T. Microbial megacities fueled by methane oxidation in a mineral spring cave. *ISME J.* **12,** 87-100 (2018).

**Figure 5 Schematic depiction of biofilm behavior under shear stress.**

Black and grey: two EPS polymers. Red dots: symbolize non-covalent bonds between polymers. Arrows: extent of shear stress

Stage 1: Biofilm with cells and EPS, undisturbed.

Stage 2: At low stress (here depicted as a shear stress, as might be imposed by a moving overlying fluid) applied to a biofilm grown in a low shear environment the biofilm can immediately stretch out and spring back when the stress is removed, pulled back by the intermolecular forces between matrix components. The biofilm behaves like a viscoelastic solid.

Stage 3: As shear increases the biofilm flows more as bonds begin to break and polymers move past each other and when the shear is removed remaining bonds slowly pull the biofilm back but it never regains its original form; patches of the biofilm can be teared off. The biofilm behaves like a highly viscous liquid.

Stage 4: After exceeding a critical shear force, many intermolecular forces are broken, the polymers are gliding past each other and the biofilm flows like a low viscosity liquid, sometimes in ripples. When the shear stress is removed the biofilm does not regain any of its form but new intermolecular bonds form providing stability to the new form.

**Box 1: Matrix dispersion and degradation: leaving the house and eating it**

The biofilm matrix is a dynamic space with continuous production and degradation of all EPS components, which turns them into a backup nutrient source. The many structural components are produced, transformed and degraded by a variety of extracellular matrix housekeeping enzymes, either produced by the producer itself or by other biofilm microorganisms176; ultimately, they can serve as ample carbon sources, as reported in activated sludge177, soil178 or in the deep sea179.

The degradation of matrix components is of particular importance for the dispersion of bacteria from the biofilm and of practical relevance for anti-biofouling measures. Many enzymes have been tested for single- and mixed biofilm removal. The mechanisms are diverse and affected by numerous factors, both intrinsic and extrinsic to the biofilm54,55. In general, the dispersion coincides with the expression of genes encoding matrix-degrading enzymes, primarily for the degradation of self-produced adhesins, DNA and polysaccharides55. Several recent examples are described, including galactosidase from *Mycobacterium*180 and glycosidase from *Desulfovibrio*181. Endonuclease is expressed to degrade the DNA present in the matrix and glycoside hydrolases for degradation of the polysaccharides Psl and Pel182. The surface-associated protein CdrA is matrix-stabilizing and when released from cells, it impedes the dispersion55. *Streptococcus* produces an arsenal of exoenzymes that serve multiple purposes such as facilitating dispersal, maintaining or changing of matrix or biofilm properties, and degrading its own or other EPS components as nutrients140.

Phages are also involved in biofilm dispersal. EPS-degrading enzymes can be encoded in bacteriophages where they are believed to facilitate the access of phages within the biofilms183. These include depolymerases degrading sialic acid, levan, xylan, dextran, rhamnogalacturonan, poly-γ-glutamate, hyaluronate, alginate, and triacylglycerol, and can, besides various biotechnological applications, also be suggested for biofilm disruption183.

Our knowledge about the degradation of polysaccharides in environmental biofilms is limited as most bacteria in these communities are uncharacterized and the structure of the polysaccharides is extremely variable33,184. Enzymes that break down glycans are called CAZymes (carbohydrate-active enzymes). The high specificity of many of these enzymes can provide information about the structure of degraded glycan184. Bacteria belonging to the phylum Bacteroidetes utilize hundreds of enzyme combinations to degrade glycans184. They encode glycan-degrading systems termed polysaccharides utilization loci (PULs), and it seems that each PUL can break down specific glycan structures184. Bacteroidetes have surface-linked carbohydrate binding proteins, sugar transporters, and a sugar sensing apparatus detecting early degradation products185. Members of the Bacteroidetes, e.g. the families *Cytophagaceae* and *Saprospiraceae*, are abundant in environmental biofilms, e.g. in soil and wastewater systems, and play an important role in the degradation of exopolysaccharides186.

Cellulose is also degraded by numerous bacteria and besides the Bacteroides, well-known biofilm bacteria such as *Bacillus*, *Acinetobacter*, *Pseudomonas* can do so, but the mechanism is best described in relation to degradation of plant material (lignocellulose)187,188.

Extracellular proteins are generally easily degraded by a few conserved families of proteases that are excreted by a variety of bacterial species, and there are reports of their successful application to eradicate specific biofilms, e.g. of *Bacillus*189. Most bacteria excrete multiple proteases. For example, *P. aeruginosa* secretes several proteases that act on both biofilm and host proteins190. Bacterial functional amyloids are characterized by their extreme robustness, also to enzyme attack, and no specialized amyloid-degrading microbial enzymes are described today. Microbial keratinases are known to also degrade beta-amyloids related to human disease191.

eDNA can be degraded by a range of extracellular nucleases, e.g. promoting the degradation of the biofilm matrix of several pathogens such as *P. aeruginosa* and *Vibrio cholerae*, and leading to dispersal of the biofilm192. In marine sediments some bacterial species seem to be specialized in degrading eDNA, some producing up to 5 different nucleases193.

**Box 2: The matrix: a safe haven for biofilm cells**

One of the defining traits of bacterial biofilms is the high degree of tolerance to antibiotics and other antimicrobial agents, regardless of the bacterial species or class of antimicrobial substances194. Usually, this tolerance is lost upon dispersal of the biofilm cells and is different from resistance, which is genetically encoded195. The matrix contributes to antimicrobial tolerance in two distinct ways, both of which provide multiple protection mechanisms for the bacteria i) by concentration gradients of antimicrobial substances due to interaction with matrix components, and/or ii) by adaptive responses of the biofilm cells.

The matrix contributes to tolerance by the establishment of diffusion-limited gradients, which are the result of chemical/enzymatic reactions of the matrix with antimicrobials, an effect termed “diffusion-reaction inhibition”196. Generally, the matrix is negatively charged due to the presence of molecules such as anionic polysaccharides, teichoic acids, acidic proteins and eDNA which can bind, e.g., cationic antibiotics such as vancomycin197 and tobramycin198. In addition, the Pel polysaccharide was shown to protect *P. aeruginosa* biofilms from aminoglycoside antibiotics by electrostatic interactions199. Matrix components can also bind host defense molecules such as antibodies, acting as a sink before they reach the cells, as was shown for IgG binding with poly N-acetyl glucosamine in a *Staphylococcus* biofilm200 and eDNA binding of human β-defensin-3, a cationic antimicrobial host defense protein201. Oxidizing disinfectants are first consumed by matrix components which then act as sacrificial material before they can act on the cells, while the underlying chemical reaction plays an important role. For example, bacterial alginate reacts with chlorine and significantly reduces its concentration. This effect is due to the acetyl substituents of alginate, and non-acetylated alginate did not neutralize the biocide202. The protective effect of the matrix can also be enzymatic; for example, catalase was shown to neutralize hydrogen peroxide in biofilms203.

For particles, the biofilm matrix may also act as a physical barrier, not just slowing down but preventing penetration. Diffusion studies and particle tracking have shown that channels within the biofilm matrix are usually around 100 to 200 nm, but sometimes even in the range of 100 µm wide174,204. Since both channels and charge are important to consider when designing delivery agents such as nanoparticle carriers174, these factors should be taken into consideration with respect to managing biofilm penetration.

On the cellular level, the response can be manifold. The heterogeneous distribution of metabolic activity could be visualized by *in-situ* stimulated Raman scattering, imaging the incorporation of stable isotopes130. These authors showed that under microaerobic conditions phenazines promoted tolerance of *P. aeruginosa* against ciproflaxin. Antibiotics at sub-inhibitory concentrations have been shown to induce matrix synthesis205 and biofilm formation in several species206. Oxygen limitation in biofilms containing aerobic or facultative aerobic species is well characterized through microelectrode studies207, since oxygen is sparingly soluble in aqueous solution and consumed by respiration faster than it can diffuse into the biofilm. Under anaerobic conditions, tolerance towards antibiotics is increased208. Acidogenic bacteria can cause the pH to drop within the biofilm209 and low pH has been associated with the more antibiotic-tolerant small colony variant formation of *S. aureus* in biofilms210. The cells within biofilms can drastically slow down their growth under nutrient limitation, preventing interference of antibiotics with metabolism211. Upon slow growth conditions, e.g., due to nutrient limitation, the cells can enter states of dormancy212 and become persisters213, or develop the newly identified antibiotic-tolerant “phoenix phenotypes”214

Transfer of resistance genes from eDNA or other cells is facilitated by high cell density in biofilms, which have been termed “hot spots” of horizontal gene transfer92,215. A further protective mechanism that the matrix provides is through providing a physical shelter from disruption whether by mechanical forces exerted, for example, by phagocytes during attempted engulfment213 or an applied fluid shear156.