Why Microorganisms Live in Biofilms and the Problem of Biofouling

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Abstract Microbial biofouling is a problem of microbial biofilms. Biofouling occurs in very different industrial fields and is mostly addressed individually. However, the underlying phenomenon is much more general and in order to understand the processes causing biofouling, it is good to understand the basics of biofilm formation and development. Almost every surface can be colonized by bacteria, forming biofilms. After adhesion, the cells embed themselves in a layer of extracellular polymeric substances (EPS), highly hydrated biopolymers of microbial origin such as polysaccharides, proteins, nucleic acids and others. In this matrix they organize their life, develop complex interactions and resistance to biocides. The resulting biofilm structure is highly heterogeneous and dynamic. It is kept together by weak physicochemical interactions of extracellular polymeric substances, which have to be overcome when cleaning is attempted. The ecological advantages for the biofilm mode of life are so strong that almost all microorganisms on earth live in biofilm-like microbial aggregates rather than as single organisms.

1 Biofouling

Slime on surfaces is the usual manifestation of a phenomenon called "biofouling". It occurs in a wide range of industrial processes and in all of them it is a nuisance, sometimes a very expensive one. It is fought against in each industrial area individually and there are many "re-inventions of the wheel" and many common mistakes – although the underlying problem is always the same: microbial biofilms. Five common mistakes in conventional anti-fouling measures can be identified in most cases are:

1. *No early warning systems*: Biofouling is detected by losses in process performance or product quality – no monitoring system.

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2. *No information on biofilm site/extent*: Sampling is performed of the water phase, which gives no information about site and extent of fouling films; sampling is not performed on surfaces.
3. *Disinfection is performed as a countermeasure*: This is not cleaning, while in most cases, the problem is caused by biomass – dead or alive. Biocides leave dead biomass on surface, providing good regrowth.
4. *No nutrient limitation is considered*: However, nutrients are potential biomass and are not reduced by biocides.
5. *No optimization of countermeasures*: Efficacy control is performed only by process or product quality – see point 1.

In very diverse industrial fields, biofouling problems all originate from the same cause: microbial biofilms. Biofilms follow common natural laws, which are important to be understood for more effective countermeasures. Basically, in biofouling the same processes occur as in biological filtration: microorganisms colonize surfaces, sequester nutrients from the water phase and convert them into metabolites and new biomass. Industrial systems frequently offer large surface areas, which invite colonization and subsequent use of biodegradable substances, leading to an extent of biofilm development that interferes with process parameters or product quality. Biofouling can be considered as a "biofilm reactor in the wrong place and at the wrong time". Therefore, detailed knowledge about biofilms is crucial for understanding and preventing biofouling as well as for successful anti-fouling measures.

The purpose of this chapter is to highlight the reasons why microorganisms form biofilms. They are the most successful form of life on earth and it is not surprising, that they cannot be eliminated easily. In many cases, microbial biofilms precede macroorganismic settlement (e.g. by larvae, barnacles and mussels), a phenomenon called macrofouling.

2 Microbial Biofilms

It is only few decades since microorganisms, sitting at the walls of microbiological liquid cultures, on rocks, sediments, in soil, on leaves, skin, teeth, implants or in wounds turned from a nuisance that could not be investigated by classical microbiological methods into a highly active field of research in which biofilms were acknowledged as the dominant form of life for microorganisms on earth (Flemming 2008). It became obvious that microorganisms on earth generally do not live as single cells and in pure cultures but do so in aggregates of mixed species. Such aggregates can consist of microcolonies as well as of patchy or confluent films on surfaces, but also as thick mats, sludge or flocks in suspension. By convention, all these phenomena are subsumed under the (somehow vague) term "biofilm" (Donlan 2002). It was just a shift of point of view that made it evident that this form of life could be found everywhere. In fact, biofilms are the first form of life recorded on earth, dating back 3.5 billion years (Schopf et al. 1983), and the most successful one. Biofilms are found even in extreme environments, such as the walls of pores in glaciers, in hot vents, under pressure

of 1,000 bar at the bottom of the ocean, in ultra-pure water as well as highly salty solutions, and on electrodes active through the entire range of thermodynamic water stability. Biofilms occur as endolithic populations in minerals, on the walls of disinfectant concentrate pipes or even in highly radioactive environments such as nuclear power plants. The surface of almost all living organisms is colonized by biofilms, which provide in many cases a protective and supportive flora (e.g. skin flora), while in other cases they cause transient, acute, chronic and even fatal diseases. Biofilms are substantially involved in the biogeochemical cycles of carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus and many metals (Ehrlich 2002). Enhancing mineral weathering processes by microbial leaching, they mobilized metal ions that were vital for further evolution. In biofilms, photosynthetic organisms evolved from originally anaerobic conditions on earth, providing oxygen as a "waste gas" from photosynthesis to the atmosphere of this planet and restricting the space for living of anaerobic organisms, which first dominated life on earth, to oxygen-depleted areas. Predation among biofilm organisms is thought to have led to endosymbionts and, eventually, to the evolution of eukaryotic organisms and the concept of infection.

One of the reasons for the late acknowledgement of biofilms is certainly the insufficient suitability of conventional microbiological methods. The introduction of fluorescence microscopy and confocal laser scanning microscopy, micro-electrodes, advanced chemical analysis with particular respect to protein analysis, and, most powerfully, molecular biology has allowed biofilm biology to be revealed in much greater detail. As a consequence, the literature in this field has virtually exploded with at least 100,000 publications on biofilms currently. The advance of knowledge is immense and fast, and this brief chapter can only superficially cover it. From a life science point of view, the most exciting aspect is that microorganisms today cannot be viewed as blind little individuals that compete as much as they can, but as complex communities with division of labour and many aspects of multicellular life (Flemming 2008). This is certainly a new understanding of microbiology with big consequences for biotechnology, medicine and handling of microbial problems in technical processes.

The biofilm mode of life provides a range of advantages to the single cell planktonic mode of life. One of the biggest advantages is the fact that the cells can develop stable interactions, resulting in synergistic microconsortia. An example is the close association of ammonia oxidizing and nitrite oxidizing bacteria. The ammonia oxidizers produce nitrite, an inhibitory end product that is comfortably used as substrate by the nitrite oxidizers. This process occurs in the environment and has been employed in nitrification steps in waste water treatment for a long time and with great success. There are many other examples of orchestrated degradation of substrates by cascades of organisms.

3 Extracellular Polymeric Substances

A characteristic feature of biofilm organisms is that they are kept together and attached to surfaces by means of their extracellular polymeric substances (EPS, Flemming and Leis 2002). An example is shown in Fig. 1, which is a scanning electron micrograph

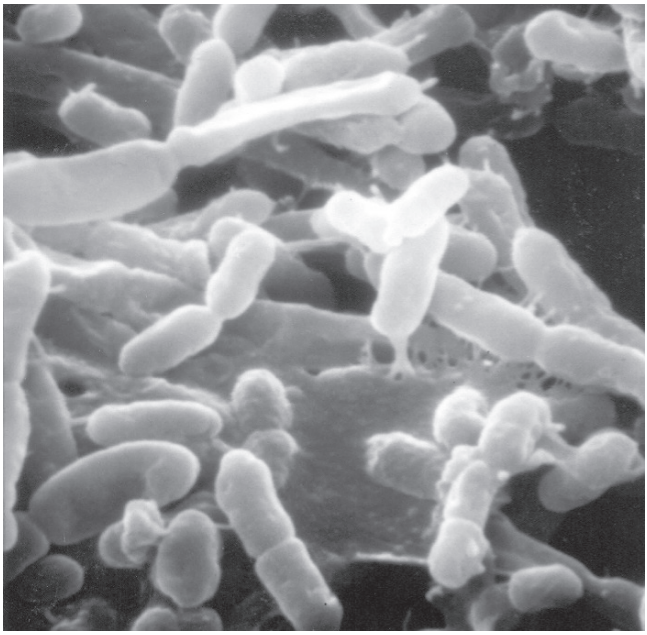

Fig. 1 Scanning electron micrograph of a biofilm of *Pseudomonas putida* on a mineral surface. EPS (dehydrated for SEM sample preparation) are surrounding the cells, keeping them together and on the surface

of *Pseudomonas putida* on a mineral surface. The sheet-like material that surrounds the cells is EPS, dehydrated by sample preparation for SEM observation.

The EPS determine the immediate conditions of life of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity and mechanical stability – all belonging to the parameters on which the conditions of life in a biofilm depend (Branda et al. 2005). This section represents a recent synopsis of the actual state of understanding of the role of EPS (Flemming et al. 2007).

EPS are biopolymers of microbial origin in which biofilm microorganisms are embedded. In fact, the biopolymers are produced by archaea, bacteria and eukaryotic microbes. Contrary to common belief, they are certainly more than only polysaccharides. Additionally, they comprise a wide variety of proteins, glycoproteins, glycolipids and in some cases surprising amounts of extracellular DNA (e-DNA). In environmental biofilms, polysaccharides are frequently only a minor component. All EPS biopolymers are highly hydrated and form a matrix, which keeps the biofilm cells together and retains water. This matrix interacts with the environment, e.g. by attaching biofilms to surfaces and by its sorption properties, which allows for sequestering dissolved and particulate substances from the environment providing nutrients for biofilm organisms. The EPS influence predator–prey interactions, as demonstrated in a system of a predatory ciliate and yeast cells. Grazing led to an increase in biofilm mass and viability with EPS as preferred food source.

Curli as proteinaceous fibrils have gained more interest beyond infection as curli-like fibrils have also been found to play an important role in natural biofilms produced by a variety of different microorganisms. An abundance of amyloid adhesions in natural biofilms has been found, which may contribute considerably to their mechanical properties. Strengthening of biofilm structure is crucial for the stability of the "house" and the continuation of synergistic interactions based on spatial proximity of various biofilm organisms.

Cellulose has been found to be a constituent EPS component in amoebae, algae and bacteria. In agrobacteria, cellulose is involved in attachment and it seems as if cellulose plays an underestimated role in environmental EPS. It is formed by a variety of organisms and influences biofilm structure. Cellulose is also important in infectious processes when co-expressed with curli fimbriae in *Escherichia coli* (Wang et al. 2007).

Biofilms are also an ideal place for exchanging genetic material and maintaining a large and well-accessible gene pool. Horizontal gene transfer is facilitated as the cells are maintained in close proximity to each other, not fully immobilized, and can exchange genetic information. Significantly higher rates of conjugation in bacterial biofilms compared to planktonic populations have been reported (Hausner and Wuertz 1999).

The EPS matrix is not only composed of a variety of components but, in addition, these are able to interact. One example is the retention of extracellular proteins such as lipase by alginate. Such mechanisms are crucial for preventing the wash-out of enzymes, keeping them close to the cells that produced them and allowing for effective degradation of polymeric and particulate material. This leads to the concept of an "activated matrix". Activation is made even more dynamic and versatile by the excretion of membrane vesicles (MVs). These highly ordered nanostructures act as "parcels" containing enzymes and nucleic acids, sent into the depth of the EPS matrix. Such vesicles, along with phages and viruses (which are of similar size), can serve as carriers for genetic material and thereby enhance gene exchange. Through their chemistry, the MVs may bind extraneous components; their enzymes may help degrade polymers, providing nutrients or inimical agents and thereby inactivating them. Furthermore, they seem to be part of the "biological warfare" within biofilms, occurring as predatory vesicles containing lytic enzymes. This biological warfare is also long-range as, in common with other matrix material, MVs are shed from the biofilm. In this respect, vesicles are "missiles" delivering, among others, virulence factors and cell-to-cell signals (Schooling and Beveridge 2006).

The composition, architecture and function of the EPSmatrix reveal a very complex, dynamic and biologically exciting view. First of all, the matrix is a network providing sufficient mechanical stability to maintain spatial arrangement for microconsortia over a longer period of time. This stability is provided by hydrophobic interactions, cross-linking by multivalent cations and entanglements of the biopolymers with e-DNA as a newly appreciated structural component. The forces that keep the biofilm matrixtogether are provided, thus by weak physicochemical interactions such as hydrogen bonds, van der Waals forces and eletrostatical interactions. They are schematically depicted in Fig. 2 (after Mayer et al. 1999).

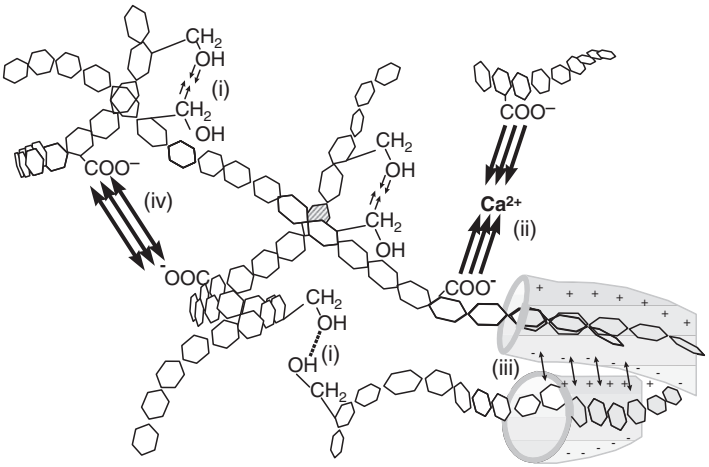

Fig. 2 Forces that keep the EPS matrix together: (**i**) hydrogen bonding, (**ii**) cation bridging, (**iii**) van der Waals forces, (**iv**) repulsive forces (after Mayer et al. 1999)

The repulsive forces are of big importance for the biofilm structure as they prevent a polymer network from collapsing. Water is equally important as it dilutes the macromolecules and limits the number of interacting groups. During desiccation, more interaction takes place and turns biofilms into practically insoluble structures (Fig. 3).

When microbial biofilms are to be removed from surfaces, as in the case of cleaning, these weak binding forces have to be overcome. Although the individual forces are low, the gross overall binding force can exceed that of covalent bonds, but it is not a directed bond. Therefore, in response to shear forces, biofilm first show characteristics of viscoelastic bodies, while when a breaking point is exceeded, they have properties of viscous liquids (Körstgens et al. 2001). Cleaning has to attempt weakening of the binding forces in order to support the efficacy of shear forces. From this point of view, it is very obvious that killing of the biofilm organisms will not contribute to cleaning unless the matrix structure is affected.

In conclusion, it seems as if "slime" has been very much underestimated and it turns out that the EPS matrix is considerably more than simply the glue for biofilms. Rather, it is a highly sophisticated system that gives the biofilm mode of life particular and successful features.

4 Structure of Biofilms

The biofilm matrix is highly hydrated and very heterogeneous. The morphology of a biofilm appears very variable. Figure 4 shows an artists view of various aspects of evolving and mature biofilms, as developed from many recent findings in biofilm research.

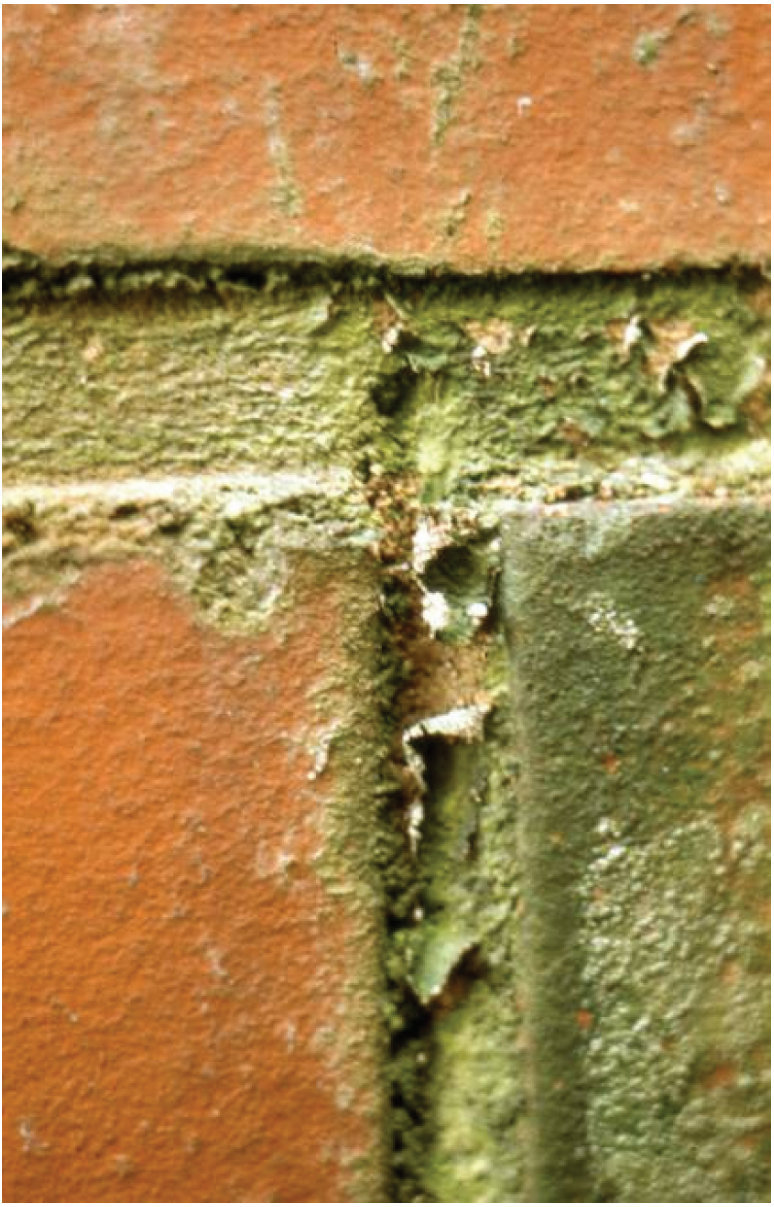

Fig. 3 Desiccated biofilm. The cohesive forces and the surface adhesion forces increase. Curling of biofilms occurs and sand grains from mortar are ripped out, contributing to microbially influenced weathering

The figure reveals structural aspects that make life in biofilms even more attractive. The porous architecture allows for convectional flow through the depth of the biofilm, while within the EPS matrix only diffusional transport is possible. Organisms

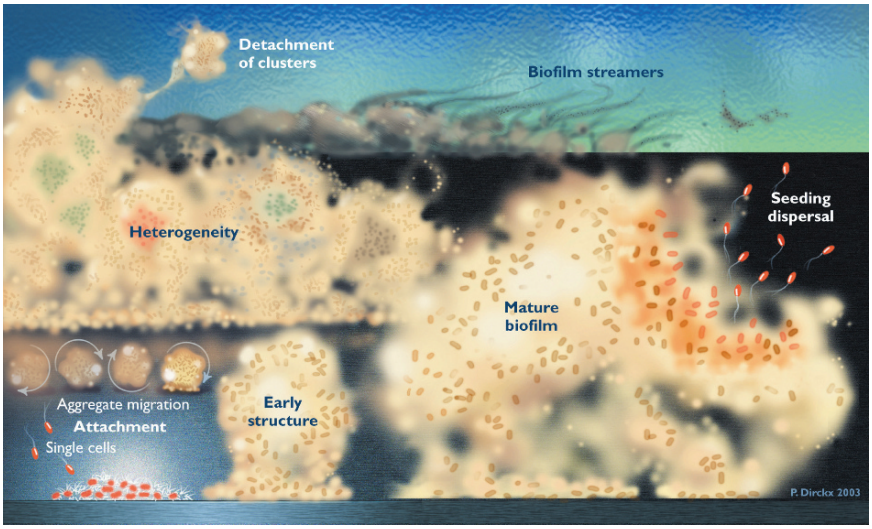

Fig. 4 Structure and processes in a biofilm (permission of Peggy Dirkx, Center for Biofilm Engineering, Montana State University, Bozeman, MT)

at the bottom of the biofilm, thus, can get access to nutrients without competition from those at the interface to the bulk water phase. Strong gradients can occur in biofilms, e.g. by actively respiring aerobic heterotrophic organisms, which consume oxygen faster than it can diffuse through the matrix. This generates anaerobichabitats just below highly active aerobic colonies in distances of less than 50 μm. Other gradients, such as pH-value, redox potential and ionic strength are known within biofilms. The result is complex interactions and a functionally structured system. The ecological relevance of this heterogeneity has inspired Watnick and Kolter (2000) to describe the biofilm as a "City of Microbes".

Another feature of biofilm cells is the increased tolerance to biocides, compared to planktonic cells (Schulte et al. 2005). It must be taken into consideration that biofilms have existed for billions of years and have survived all kinds of adverse conditions. Therefore, many different mechanisms have evolved for resistance, and they are far from being fully understood (Lewis 2001). The fact is that resistance genes can be exchanged and that biofilms have been observed even in disinfection concentrate pipes. The resistance of biofilms is particularly problematic in medicine where contaminations of implants, catheters or bones result in long-term infections, which in many cases can only be overcome by radical measures such as exchange of implants and removal of bone parts. In drinking water systems, biofilms can harbour hygienically relevant organisms that may even proliferate if nutrients are provided. Even enhanced application of disinfectants such as chlorine will not eradicate such biofilms.

5 Ecological Advantages of the Biofilm Mode of Life

From the above highlighted context, it is obvious that microorganisms gain clear advantages from the biofilm mode of life. This has been summarized very well by Costerton (2007), a biofilm pioneer. The ecological advantages of the biofilm mode of life are quite a few more and can be summarized as follows:

- Formation of stable microconsortia
- Biodiversity: gradients create different habitats
- Gene pool and facilitated genetic exchange
- Retention of extracellular enzymes in the matrix
- Access to particulate biodegradable matter by colonization
- Recycling of nutrients because lysed cells are retained in the biofilm
- Protection against biocides and other stress
- High population density: threshold concentration of signalling molecules is easily reached, facilitating intercellular communication

These are good reasons explaining the preference for the biofilm mode of life of most microorganisms on earth.

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