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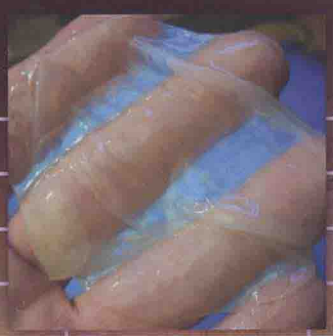

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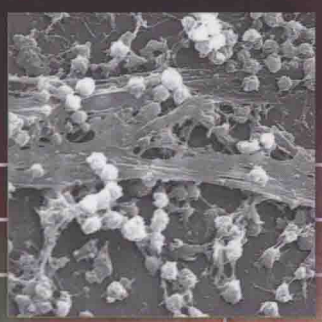

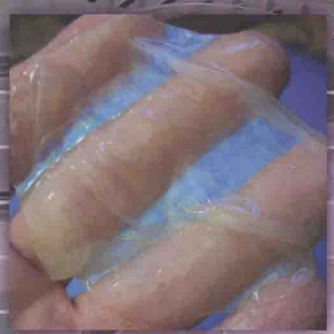

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Foreword

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Biofilm science is a relatively new technical discipline focused on the understanding and modulation of the combination of biological and chemical processes as well as on the transport and interfacial processes that potentially affect the microbial accumulation and activity on both biotic and abiotic surfaces. Research on biofilms has progressed rapidly in the last decades. Due to the fact that biofilm studies required the development of new analytical tools in the experimental and the computer science fields, many recent advances have resulted from collaboration between microbiologists, microbial ecologists, medical doctors, pharmacologists, engineers and mathematicians. The scientific community has come to understand much more about the particular aspects of microbial biofilms through the use of a variety of microscopy and other physical methods, as well as new chemical, and molecular biology approaches [7].

Biofilms have been first documented in 1943 by Zobell, who reported the attachment of layers of microbial cells to bottle walls and the increase in the biological activity of batch suspended cultures when glass rods were added [22]. Further investigations revealed that this effect was even more pronounced under oligotrophic conditions when compared to the results obtained under high nutrient conditions [11, 22]. These conclusions emphasized the perception that the adhesion is a strategy of microorganisms to access nutrients from the surface and biofilm. The study of Characklis [5], about microbial slimes in industrial water systems, revealed their high cohesiveness as well as their strong resistance to disinfectants, but it was Costerton et al., [6] in 1978 who postulated the general theory of biofilm predominance. Only recently, however, have attempts been made to define genetic, physiological and ecological basis of such phenomena [2, 8, 14].

It is supposed that biofilm is the first form of communitarian life recorded on the planet, being estimated that most microorganisms on Earth are organized in biofilms and they even occur in extreme environments such as hydrothermal vents, nuclear power plants and disinfection pipelines [6].

Biofilms are formed ubiquitously in any interface (liquid or solid) in contact with water. The ubiquity of biofilms can cause significant problems of public health, medicine and industry concern [6, 9, 10, 15]. Accordingly, there has been a great deal of research to better understand biofilm development and to identify improved control strategies. In the health context, some diseases and adverse medical conditions are now recognized to be the result of a biofilm infection.

Biofilms are as versatile as they are ubiquitous. Intentional and unintentional biofilms concern a broad range of areas, comprising special attention in the industrial, environmental and biomedical areas [3, 4]. Biofilms can be beneficial or detrimental depending on where they are found. Biofilms used in pharmaceutical and food fermentation industries, wastewater treatment plants and natural biofilms present in lakes or rivers (which contribute to pollutant degradation) are examples of beneficial biofilms. On the other hand, biofilms that accumulate in cooling water towers and heat exchangers, membrane systems, filters, drinking water distribution systems, swimming pools, food processing equipment, paper manufacture industries, ship hulls, catheters, medical implants, tissues, teeth, lungs and contact lenses are harmful. One of the main problems of these biofilms is their potential impact in human health. Moreover, they may create problems for hygiene and cleaning, as well as being responsible for energy losses in heat transfer equipment, blockages in flow systems and microbial induced corrosion.

In engineered systems, detrimental biofilm formation is usually named as "biofouling" or "biological fouling". Its costs have not been fully assessed, but dispersed data from different case studies suggest a quite significant financial burden brought upon by biofouling. According to Koch et al., [12] and Wang [21], microbial influenced corrosion accounts for about 20-30% of all corrosion costs in the USA, the latter representing around 3% of the county's Gross National Product (GDP). Macrobiological fouling layers (barnacles, oysters, algae, etc) attach to biofilms on ship's hulls, substantially increasing the drag forces against motion of the vessel. These layers may cause up to 30%-40% higher fuel consumption. Additionally, in order to mitigate biofouling unwanted effects, the sea water cooled industrial equipment operators (including thermal power plants) all over the world are estimated to spend around 10-15 billion dollars/year [17]. Azis et al., [1] indicates that the costs of biofouling in desalination plants may amount to 15 billion US$/year worldwide.

The general fouling costs (all types of fouling phenomena) have been estimated in several countries to represent 0.25% of their respective GDP-Gross National Product [13]. In view of the values reported above for biofouling costs in a diversified number of cases, a conservative educated guess would point out to biofouling costs being at least 20% of the overall fouling costs. Since the world GDP is around 71×10^{12} US$ [16], the total biofouling costs may reach 3.5×10^{10} US$/year worldwide.

Despite the huge economical losses entailed with biofilm formation in industrial or engineered systems, medical biofilms usually have a worse reputation due to the simple fact that biofilms can kill. It has been estimated that more than 60% of all microbial infections in humans are caused by biofilms [18] and that a significant part of these incidences corresponds to hospital-acquired infections that have been estimated to be greater than 2 million per year in American hospitals alone [17]. In fact, hospital-acquired infections have been considered the fourth leading cause of death in the US affecting about 10% of the hospital patients [17]. About 60-70% of the hospital-acquired infections are related to implanted medical devices that have become indispensable in modern-day medicine [17]. More than 5 million central

venous catheters are inserted annually in the US and it expected that 2-12% will results in sepsis [19]. The cost per infection is estimated to be between $34,000 and $56,000 and the annual cost of caring for patients with catheter-associated bloodstream infections can reach $2.3 billion [19]. Another common biofilm-related infection results in ventilator associated pneumonia. It has been estimated that this condition alone is responsible for an expenditure of $1.5 billion per year corresponding to 20-34% of all hospital costs [20]. The problem becomes even more serious if one starts to consider the effects of antibiotic resistance which is more likely to occur in biofilms than in planktonic cells. In fact, it is calculated that infection treatment of resistant organisms costs twice as much as treating those that are susceptible to conventional treatment. It has been estimated that, for an average 500-bed hospital, the additional cost for treatment of biofilm infections that have developed some sort of antibiotic resistance would be up to $21 million (considering only sepsis, pneumonia, and cervical site infections) and that approximately 120 patients would die from these infections [20].

Having considered the economical and societal impacts of biofilms it is clear that biofilm control is a question of paramount importance. However, biofilm control strategies that either potentiate their development in beneficial applications or inhibit/eliminate them in detrimental cases have to be designed taking into consideration many factors, among which the definition of acceptable biofilm threshold levels. As it will be detailed in the following chapters, biofilms affect many aspects of our life. In some of these aspects, the presence of a biofilm impacts on the performance of a given system and therefore biofilm development to a certain level is tolerated (for instance in cooling water systems) or even promoted (in biofilm reactors). There are other scenarios, particularly in the health and food sectors, where biofilms have to be eliminated because they are simply too much of a risk. In these latter cases we are at war with these biofilms. In this respect, the information presented in the following chapters is intended to provide clues towards a better understanding of the biofilm development process that may eventually make us win some of these battles.

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Contents

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Chapter I

Biofilm Formation, Development and Relevance

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Abstract

Bacteria are able to grow adhered to almost every surface, forming architecturally complex communities termed biofilms. Biofilms are multicellular communities held together by a self-produced extracellular matrix. Biofilm development is a dynamic process that occurs in different sequential phases and involves a series of steps. Biofilms impact humans in many ways as they can form in natural, medical, and industrial settings. For instance, formation of biofilms on medical devices, such as catheters or implants often results in difficult-to-treat chronic infections. Not all biofilms cause problems both in the clinical and environmental context. Within humans, bacterial biofilms may also play a protective role and promote a probiotic effect when consumed through various fermented foods. Also within the industrial context there are several successful examples of the positive use of beneficial biofilms. Recent data concerning biofilm formation, evolution, structure and functions are reported in this chapter. Particular emphasis is given to discussing the different adverse effects and beneficial aspects of biofilms.

Keywords: Biofilm structure, development and propagative mechanisms; Adverse and beneficial aspects

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Introduction

Recent advances in microscopy and molecular technologies have made possible the direct observation of a wide variety of natural habitats, demonstrating that in natural, industrial and medical setting, the majority of microbes are associated with surfaces within complex bacterial communities (biofilms) and not as free-floating organisms [1]. These microbial structures occur in nearly every moist environment where sufficient nutrient flow is available and surface attachment can be achieved. Biofilm is usually defined as organized communities of microorganisms attached to a surface and embedded in a matrix of self-produced extracellular polymeric substances (EPS) [2, 3]. The new technologies have made possible to examine in detail such communities, and have led researchers to conclude that biofilms represent biological systems with a high level of organization where bacteria form structured, coordinated, functional communities. Consequently, the definition of biofilm has evolved over time, taking into consideration not only the morphological characteristics, but also other physiological attributes of these biofilm-forming organisms. For example the gene expression profiles of bacteria in these microbial consortia are quite different compared with the expression profiles of the same strains grown planktonically, and such genes include those responsible for regulation and/or expression of surface adhesion proteins, appendages such as fimbriae, pili or flagella, etc. Therefore, the useful definition of a biofilm is "microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription".

Advantages of Forming a Biofilm Versus Living As Individual Cells

Biofilm is an ancient prokaryotic adaptation [5] and represents a mode of growth that allows bacteria to survive in hostile environments and to colonize new niches. Because of their dynamic character, biofilm communities can continuously change in time and space, providing better survival and growth of the associated microorganisms. For this reason, in most natural environments biofilms are the prevailing microbial lifestyle.

Environmental advantages. Protection from the environment, nutrient availability and metabolic cooperativity and acquisition of new genetic traits are some reasons why the biofilm strategy has been adopted by so many microbes [6]. Within this structure the microorganisms are protected from chemical and natural antibacterial substances, environmental bacteriophages, and phagocytic amoebae. For example, after treatment with antibiotics or chemicals, biofilm cells survive and are often responsible for recurring symptoms and medical treatment failure [8]. Some phenomena are postulated to contribute to the biofilm defence, including incomplete antimicrobial penetration, slow or no growth of some of the biofilm cells, and the expression of biofilm-specific phenotypes [9, 10].

Phenotypic variation. The properties of biofilm cells have been shown to be distinct from their planktonic counterparts. There is evidence that gene expression may be regulated once a

cell comes into contact with a surface. This up- and downregulation of genes has been demonstrated in *Pseudomonas aeruginosa* with genes associated with alginate synthesis (algC up-regulation) and in *Staphylococcus aureus* for genes encoding enzymes involved in glycolysis [11]. In some organisms, up to 22% of genes were up-regulated in the biofilm state and 16% were down-regulated [12]. Horizontal gene transfer in biofilm organisms by conjugation, transduction and transformation is also important for the evolution and genetic diversity of natural microbial communities [13,14,15]. With the cells embedded in a polysaccharide matrix, biofilms are an ideal place for exchanging genetic material at a greater rate than cells in the planktonic phase [16,17]. A biofilm community provides an ideal niche for transfer of mobile genetic elements (e.g., plasmids) that might encode beneficial traits, such as resistance to antibiotics and/or heavy metals. Conjugative plasmids might also enhance biofilm formation because plasmids repressed for horizontal gene transfer retained the ability to form biofilms even if the biofilm population included plasmid-free recipient cells. Plasmid recipient strains have been shown to demonstrate increased biofilm growth [18]. Conjugative transposons are probably the most promiscuous of all mobile elements. These elements integrate into the host cell genome and do not need to provide their own replication machinery or rely upon interactions with the host cell for stable replication and maintenance. Roberts et al., [17] investigating the horizontal gene transfer of a conjugative transposon Tn*5397* (tetracycline resistant) in a mixed species oral biofilm confirmed the transfer of genes in biofilm communities among related or unrelated strains. Virus-mediated transduction is another mode of gene transfer. Both chromosomal and plasmid DNA can be successfully transduced in natural water environments ranging from sewage plants to rivers and lakes and has been shown to confer increased survival mechanisms (e.g. antibiotic resistance) on the biofilm cells [19,20]. It was recently demonstrated that high transduction frequencies can also occur in marine environments [21], and therefore the impact of transduction on gene exchange in biofilms may be significant. Bacteriophage-mediated transfer of tetracycline resistance has been demonstrated in *S. aureus* biofilms [22], and other examples include the transfer of penicillin binding proteins (PBP) and IgA proteases [23,24,25]. Lastly, natural competence is a pervasive phenomenon among bacteria. Hence, it could be expected that transformation of DNA would occur efficiently in dense bacterial populations such as biofilms that are regulated by quorum sensing. *Streptococcus mutans* cells grown in a biofilm were transformed at a frequency up to 4×10^{-3} per cell and, in most cases, at rates of ten to 600-fold higher than planktonic *S. mutans* cells [26].

Biofilm Structure and Functions

The application of confocal scanning laser microscopes (CSLM) to biofilm research has revealed the elaborate three-dimensional structure. It is now recognised that biofilms are heterogeneous, structurally organised microbial communities containing microcolonies encased in an EPS matrix and loosely connected to nearby microcolonies by water channels and voids [27,28], these last responsible for transport of nutrients, oxygen, genes and even antimicrobial agents [4].

Microcolonies. The microcolonies that constitute the biofilm can be composed of single-species populations or multimember communities of bacteria, depending on the

environmental parameters under which they are formed [29]. Having docked at a surface, cells begin to grow relatively quickly and form a microcolony. At this stage, as the microcolonies dispersed over the surface begin to grow, first of all in the horizontal mode across the surface, and then out into the liquid phase, the three-dimensional, multispecies consortia biofilm begins to develop. In addition, secondary colonisers, i.e. those that lack the ability to initially attach to a surface, may preferentially attach to the primary colonisers by co-adhesion and contribute to community development resulting in a biofilm with a thickness in the range of 30–50 μm.

EPS. Biofilms are mainly composed of bacterial cells, organized in microcolonies, and EPS, microbial products located on or outside the cell surface. If biofilms can be metaphorically called the "city of microbes" [30], the EPS represent the "house of the biofilm cells" [31]. The production of extracellular matrix appears to overlap with all stages that occur after initial surface adhesion, but it is considerably more than simply the glue for biofilms. The EPS matrix is a dynamic system, constructed by the organisms and responding to environmental changes. It enables the cells to function in a manner similar to multicellular organisms [32]. In fact, 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 [33]. Rather, it appears to be a highly sophisticated system, which endows the biofilm mode of life with particular, successful features [31]. Given the great diversity of microbial communities that form biofilms, as well as the different environments that they inhabit, it is difficult to make generalizations about biofilm EPS structure and physiological function. Detailed composition analysis of an EPS is difficult, as EPS is often a complex mixture of polysaccharides, proteins, glycoproteins, and glycolipids, DNA, and humic acid substances [34]. Furthermore, even though carbohydrates have been identified as one of the major components of EPS, the biochemical properties of these compounds remain elusive due to their complex structures and unique monomer linkages [35,36,37,38]. The nature of carbohydrates within EPS is a dynamic function of the microbial community composition; this is reflected by the complexity of carbohydrates containing diverse sugar residues extracted from biofilm matrices [36,38,39]. The biofilm matrix not only facilitates structural organization and protects the microbial community [40], but also influence predator-prey interactions, as demonstrated in a system consisting of a predatory ciliate and yeast cells, in which grazing led to an increase in biofilm mass and viability, with EPS as the preferred food source [41]. Several studies indicate that EPS are not necessarily required for the initial attachment of microbial cells to surfaces [42], but their production is essential for the development of the biofilm matrix, providing the framework into which microbial cells are inserted [43]. EPS can account for 50–90% of the total organic carbon of the biofilm and is highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding [44]. This hydrated EPS will protect the biofilm from desiccation. Briefly, EPS can be considered the primary matrix material of the biofilm that serves as a storage facility for nutrients and can entrap other microbes and non-cellular materials, such as minerals, crystals and corrosion products.

Interstitial water channels. It has been widely reported that biofilms consist of clumps of cells separated by interstitial voids and channels [45]. The interstitial voids or channels are the lifeline of the system, since they provide a means of circulating nutrients as well as exchanging metabolic products with the bulk fluid layer [46], and it was also discovered that water could flow through the channels [47]. Probably the channels are a vital part of the