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Microbial Metropolis - A Microbial Biofilm Community

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Abstract: ***Microorganisms can form tightly bind communities such as biofilms. Many others include marine snow, anaerobic digester granules, the ginger beer plant, bacterial colonies, effluent treatment floc and food associated systems. Biofilm-associated cells can be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up and down regulation of specific genes. The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood etc.) provides an ideal environment for the attachment and growth of microorganisms (Gillings et.al.,2008). Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum and cell surface. An established biofilm structure comprises microbial cells and EPS, has a defined architecture and provides an optimal environment for the exchange of genetic material between cells. (Sur, 2008). As the origins of EPS are complex, many factors could influence the production of EPS. In future the roles of EPS components become known, identification of such key factors would then be very useful to manipulate the EPS compositions and contents in microbial aggregates and thus to improve the functions of microbial aggregates, e.g., flocculation, settlement and dewatering abilities.***

Keywords: biofilms, exopolysaccharide, conditioning films, substratum effect

1. Introduction

Until near the end of the last century microbiology was pretty simple. Everyone knew that bacteria were single-celled organisms that existed as planktonic cultures in laboratories all over the world. The research weapons of choice were bench-top fermenters operating in batch mode, or the much more powerful, continuous culture systems. Finally the penny dropped and phenomena staring microbiologists in the face received the attention they warranted, in so doing opening up a mass of fascinating new knowledge. The collective noun for the associations seen between monospecies or multi-species associations of microorganisms in general is “biofilm”, a name given to the phenomenon by Costerton et al. (1978). It is a convenient term, easy to remember, and superficially comprehensible. Biofilm suggests a substantially two-dimensional layer of living creatures associated with a surface. There are numerous examples of biofilms. A slimy layer of material associated with rocks in flowing streams or with the waste pipe from a kitchen sink, fouling on the hulls of boats and ships, dental plaque on tooth surfaces, biomass associated with trickling filters in activated sludge plants etc. All are associated with attachment to an inert surface of cells receiving nutrients from the environment above them. Some can of course also interact with the substratum on which they grow. Examples here are bacteria generating hydrolytic enzymes and degrading cellulosic substrates in the rumen of cattle, or bacterial colonies growing on gel-stabilized media containing nutrients. Unfortunately use of the word biofilm has led in general to us ignoring other interacting communities that are not surface associated, but which share many of the properties of biofilms. A few examples make this clearer. Fungi form mycelial balls when cultured in fluid media as do some bacteria cultured in semi-solid media. Beverage associations such as kefir, the ginger beer plant and kombucha all consist of yeast and lactic acid bacteria forming solid aggregates. Marine snow forms around detritus in the oceans whilst activated sludge generates a flocular or granular material.

2. Difference between Planktonic or Sessile

Microbes can be free-living (planktonic), often motile, subsisting in a homogeous liquid (in nature almost always aqueous). Others are found attached to solid surfaces (sessile) such as rocks in streams. There are advantages to both modes of life and most sessile species can generate planktonic forms that are liberated into the environment. Attachment is an effective strategy if a source of nutrient is flowing past so that growth substrates can be abstracted from the aqueous phase, whilst potentially toxic growth products are washed away. Attachment allows communities to form so that the net growth of the association is more than that of all the individual members. What is more, attached structures are more resilient, can resist antimicrobial agents, and are more able to reap the benefits of interspecies cooperation than is possible with planktonic cells. Some of the differences between the two are indicated in Table 1.

Table 1: Some differences between sessile and planktonic microbes. (Shemesh et al., 2007)

Properties	Planktonic Mode	Sessile Mode
Dominant State	Free Living	Attached
Motility	Motile	Non Motile
Dependency	Independent	Dependent
Extracellular polymers	Few or none	Extensively produced
Adhesive components	No	Yes
Matrix components	No	Yes
Quorum sensing	No	Yes
Expressing virulence	No	Yes
Produce extracellular enzymes	No	Yes
Environment	Homogeneous	Heterogeneous
Horizontal Gene Flow	No	Yes

3. Biofilm Formation

3.1 A Historical Basis

A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. Heukelekian and Heller observed the "bottle effect" for marine microorganisms, i.e., bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach. Two major thrusts in the last decade have dramatically impacted our understanding of biofilms: the utilization of the confocal laser scanning microscope to characterize biofilm ultrastructure, and an investigation of the genes involved in cell adhesion and biofilm formation.

3.2 Biofilm Defined

A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Non cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from an industrial water system and a medical device, respectively. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organism and the associated extracellular polymeric substance (EPS) matrix.

3.3. Attachment

The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface. Each of these factors will be considered in detail.

3.4. Substratum Effects

The solid surface may have several characteristics that are important in the attachment process. Characklis et al., noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals. Even though results of these studies have at times been contradictory because no standardized methods exist for determining surface hydrophobicity, some kind of hydrophobic interaction apparently occurs between the cell surface and the substratum that would enable the cell to overcome the repulsive forces active within a certain distance from the substratum surface and irreversibly attach.

3.5 Conditioning Films

A material surface exposed in an aqueous medium will inevitably and almost immediately become conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment. Loeb and Neihof were the first to report the formation of these conditioning films on surfaces exposed in seawater. These researchers found that films were organic in nature, formed within minutes of exposure, and continued to grow for several hours. The nature of conditioning films may be quite different for surfaces exposed in the human host. A prime example may be the proteinaceous conditioning film called "acquired pellicle," which develops on tooth enamel surfaces in the oral cavity. Pellicle comprises albumin, lysozyme, glycoproteins, phosphoproteins, lipids, and gingival crevice fluid; bacteria from the oral cavity colonize pellicle- conditioned surfaces within hours of exposure to these surfaces. Mittelman noted that a number of host-produced conditioning films such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions influence the attachment of bacteria to biomaterials. Ofek and Doyle also noted that the surface energy of the suspending medium may affect hydrodynamic interactions of microbial cells with surfaces by altering the substratum characteristics.

3.6 Hydrodynamics

In theory, the flow velocity immediately adjacent to the substratum/liquid interface is negligible. This zone of negligible flow is termed the hydrodynamic boundary layer. Its thickness is dependent on linear velocity; the higher the velocity, the thinner the boundary layer. The region outside the boundary layer is characterized by substantial mixing or turbulence. For flow regimes characterized as laminar or minimally turbulent, the hydrodynamic boundary layer may substantially affect cell-substratum interactions. Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface will depend largely on the velocity characteristics of the liquid. Under very low linear velocities, the cells must traverse the sizeable hydrodynamic boundary layer, and association with the surface will depend in large part on cell size and cell motility. As the velocity increases, the boundary layer decreases, and cells will be subjected to increasingly greater turbulence and mixing. Higher linear velocities would therefore be expected to equate to more rapid association with the surface, at least until velocities become high enough to exert substantial shear forces on the attaching cells, resulting in detachment of these cells. This finding has been confirmed in studies by Rijnaarts et al., and Zheng et al.

3.7 Characteristics of the Aqueous Medium

Other characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems. This effect may be due to water temperature or to other unmeasured, seasonally affected parameters. Fletcher found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces. Cowan et al. showed in a laboratory study that an increase in nutrient concentration correlated with an increase in the number of attached bacterial cells.

3.8The Established Community: Biofilm Ecology

The basic structural unit of the biofilm is the microcolony. Proximity of cells within the microcolony (or between microcolonies) provides an ideal environment for creation of nutrient gradients, exchange of genes, and quorum sensing. Since microcolonies may be composed of multiple species, the cycling of various nutrients (e.g., nitrogen, sulfur, and carbon) through redox reactions can readily occur in aquatic and soil biofilms.

3.9 The matrix: Biofilm Structure

3.9.1. Extracellular Polymeric Substances:

Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers the anionic property. This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm. In the case of some gram-positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. Hussain et al., found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins.

EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic. EPS may also vary in its solubility. Sutherland noted two important properties of EPS that may have a marked effect on the biofilm. First, the composition and structure of the polysaccharides determine their primary conformation. For example, many bacterial EPS possess backbone structures that contain 1,3- or 1,4-□-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble. Other EPS molecules may be readily soluble in water. Second, the EPS of biofilms is not generally uniform but may vary spatially and temporally. Leriche et al., used the binding specificity of lectins to simple sugars to evaluate bacterial biofilm development by different organisms. These researchers' results showed that different organisms produce differing amounts of EPS and that the amount of EPS increases with age of the biofilm. EPS may associate with metal ions, divalent cations, other macromolecules (such as proteins, DNA, lipids, and even humic substances).

EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote EPS synthesis. Slow bacterial growth will also enhance EPS production. Some of the function of EPS are listed table 2. Because EPS is highly hydrated, it prevents desiccation in some natural biofilms. EPS may also contribute to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents.

Table 2: EPS Functions

Effect of EPS	Nature of EPS component	Role in biofilm
Constructive	Neutral polysaccharide Amyloids	Structural components
Sorptive	Charged or hydrophobic polysaccharide	Ion exchange, sorption
Active	Extracellular enzymes	Polymer dedradation
Informative	Lectins, Nucleic acids	Specificity, recognition Genetic information Structure
Nutritive	Various polymer	Sources of C,N,P

3.9.2 Mechanism of Microbial Aggregates

1) Adhesion
2) Role of Holdfasts structures in biofilm
3) Coaggregation

Adhesion

The microbial surface properties are important to the interfacial interactions between the cells and the solid surface, which is of crucial importance for biofilm formation in the aquatic environment. The adsorption of EPS to a material surface would alter the substrata physicochemical characteristics and hence influence the initial bacterial adhesion process (Gomez-Suarez et al., 2002; Omoike and Chorover, 2006). In the presence of microbes (inevitable in most habitats), they will adhere to form a loose association with the surface which, after a period, becomes a strong association as specific adhesion is mediated by adhesive cell products generated by the cells. One of the most interesting and ubiquitous microbial assemblages is dental plaque. As a biofilm it can contain around 500 separate species, the majority (60–80%) of which have been isolated and studied. An excellent review paper (Rickard et al., 2008) which concentrates on communication in the plaque system, also describes early events in colonizing "professionally" cleaned tooth surfaces. Three types of binding seem to occur: (a) that between certain species and the clean surface; (b) that between cells and the conditioning film that forms and consists in the mouth of salivary components like salivary proteins, glycoproteins, and polysaccharides and (c)

coaggregation events in which different bacterial species can attach to other species including those associated with surface components (fig 1). Gibbons and Nygaard (1970) were the first to describe the coaggregation phenomenon and since then there have been numerous reports for example by Paul Kolenbrander (Kolenbrander and Williams, 1981; Kolenbrander, 1988; Kolenbrander et al., 2006, 2007).

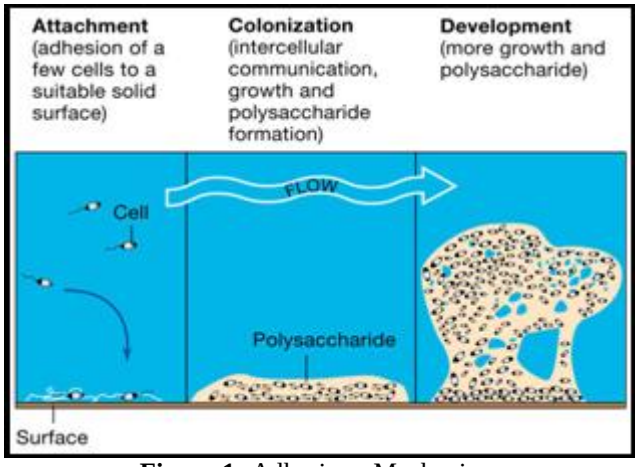

Figure 1: Adhesion –Mechanism

Role of Holdfasts structures in biofilm:(Jenal,2009)

Caulobacter crescentus is a Gram-negative, oligotrophic bacterium widely distributed in fresh water lakes and streams. *Caulobacter* is an important model organism for studying the regulation of the cell cycle, asymmetric cell division, and cellular differentiation. *Caulobacter* daughter cells have two very different forms. One daughter is a mobile "swarmer" cell that has a single flagellum at one cell pole that provides swimming motility for chemotaxis. The other daughter, called the "stalked" cell has a tubular stalk structure protruding from one pole that has an adhesive holdfast material on its end, with which the stalked cell can adhere to surfaces (fig 2). Swarmer cells differentiate into stalked cells after a short period of motility. Chromosome replication and cell division only occurs in the stalked cell stage. Its name is due to the fact that it forms a crescent shape; crescentin is a protein that imparts this shape.

The *Caulobacter* stalked cell stage provides a fitness advantage by anchoring the cell to surfaces to form biofilms and or to exploit nutrient sources. Generally, the bacterial species that divides fastest will be most effective at exploiting resources and effectively occupying ecological niches. Yet, *Caulobacter* has the swarmer cell stage that results in slower population growth. The swarmer cell is thought to provide cell dispersal, so that the organism constantly seeks out new environments. This may be particularly useful in severely nutrient-limited environments when the scant resources available can be depleted very quickly. Many, perhaps most, of the swarmer daughter cells will not find a productive environment, but the obligate dispersal stage must increase the reproductive fitness of the species as a whole.

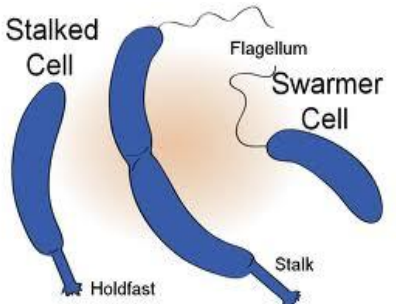

Figure 2: Holdfasts structures in biofilm

Coaggregation

More recently, coaggregation has been shown not to be the sole province of the animal mouth but communities engaging in this trick have been found in natural water samples (Rickard et al., 2002, 2003a,b). Thus, 19 distinct heterotrophic bacteria were isolated from a freshwater biofilm. Distantly and closely related strains coaggregated at inter-and intrageneric, but also at species level. So coaggregation is probably a widely distributed function of microbial communities though originally recognized and investigated most in oral ecosystems. Microbes associate with other organisms to form a range of structures from simple to quite complex. It was first reported in dental plaque (Gibbons and Nygaard, 1970; Gibbons and Houte, 1975).

4. Importance of Biofilm

4.1 Quorum sensing

Cell-to-cell signaling has recently been demonstrated to play a role in cell attachment and detachment from biofilms. Bacteria that use quorum sensing constitutively produce and secrete certain signaling molecules(called *autoinducers* or *pheromones*). These bacteria also have a receptor that can specifically detect the signaling molecule (inducer).When the inducer binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. Thus, in order for gene transcription to be activated, the cell must encounter signaling molecules secreted by other cells in its environment.Activation of the receptor induces the up-regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time. This coordinated behavior of bacterial cells can be useful in a variety of situations (fig 3).

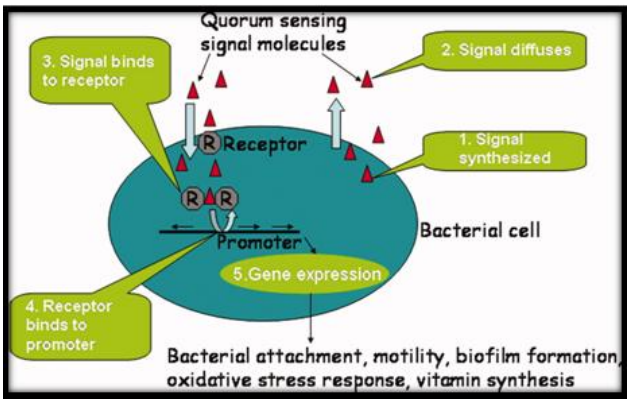

Figure 3: Schematic representation of Quarum sensing

4.2. Bacterial Cross-talk

Most bacterial communities consist of numerous, different bacterial species. Although many different homoserine lactone autoinducers are known, inevitably many bacteria produce the same versions and cross-talk can occur between them. For example P. aeruginosa, Serratia liquefaciens, and Aeromonas hydrophylla all produce N-butanoylhomoserine lactone. In mixed biofilm cultures of P. aeruginosa and Burkholderia cepacia, the latter could respond to HSLs produced by the pseudomonad though the traffic was one way only (Williams, 2007). Another form of intercellular communication is between Gram-positive bacteria including Bacillus subtilis to help regulate horizontal gene transfer.

4.3 Anti microbial resistant:

The production of an exopolysaccharide matrix, or glycocalyx, is one of the distinguishing characteristics of biofilms. It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. In this review highlight a few of the more recent studies on the subject of antibiotic diffusion through a biofilm. Although mathematical models suggest that, for many antibiotics, there should be no barrier to their diffusion into a biofilm, some studies have shown an apparent failure of certain antimicrobial agents to penetrate the biofilm. Chlorine, a commonly used disinfectant, did not reach 20% of the bulk media's concentration within a mixed *Klebsiellapneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode. In fact, the penetration profile was suggestive of a substrate being consumed within the matrix. Suci *et al.* used infrared spectroscopy to show that the rate of transport of the antibiotic ciprofloxacin to the surface of a colonized surface was reduced compared with transport to a sterile surface. These authors suggested that the ciprofloxacin was binding to the biofilm components. Other groups have taken different approaches to address the question of whether the biofilm acts as a barrier to antimicrobial agents.

On the one hand, *P. aeruginosa* biofilms were formed on one side of a dialysis membrane and the amount of piperacillin that penetrated the biofilm was measured. Consistent with the results discussed above, the *P. aeruginosa* biofilm prevented diffusion of this antibiotic. On the other hand, *Staphylococcus epidermidis* biofilms formed in a similar manner allowed for the diffusion of rifampicin and vancomycin across the membrane, implying that these antibiotics could efficiently penetrate this biofilm. These results suggest that inhibition of diffusion cannot always explain resistance to antimicrobial compounds. A difference between thick and thin biofilms and their resistance to antibiotics has been observed. Penetration of a thin biofilm-covered bead [average cell density ~3.5 log colony-forming units (cfu) cm−2] by hydrogen peroxide was observed directly, even though the cells within the biofilm were more resistant to the compound compared with planktonic cells15. By contrast, thicker biofilms, grown on glass slides (average cell density ~7.6 log cfu cm−2), presented a barrier to the penetration of hydrogen peroxide. Interestingly, hydrogen peroxide was able to penetrate a thick biofilm formed by a mutant strain of *P. aeruginosa* that lacked one of the major catalase genes, *katA*. As catalases are enzymes that neutralize hydrogen peroxide, this result suggested that, in thick biofilms, cells were protected from hydrogen peroxide penetration by the catalasemediated destruction of this compound.

Anderl *et al.* formed *K. pneumoniae* colony biofilms on agar plates with or without antibiotic. By placing a filter at the top of the colony, essentially sandwiching the colony, they were able to assay directly for antibiotic diffusion from the agar plate through the colony by performing a standard zone of inhibition assay with the filter. This breakthrough study showed that ampicillin was unable to penetrate the biofilm and that the production of the ampicillindegrading enzyme □-lactamase was responsible for this phenomenon, as the ampicillin was able to penetrate a biofilm formed by a □-lactamase mutant. Surprisingly, the □-lactamase mutants grown in a biofilm were still resistant to ampicillin, suggesting that other mechanisms contribute to the resistance of these cells. Furthermore, ciprofloxacin was able to penetrate the biofilm, yet, as was the case with ampicillin, it was unable to kill the biofilm bacteria. This simple method allowed for the differentiation between transport effects and other mechanisms and thus provides a powerful tool for the further analysis of the molecular mechanism of biofilm resistance to antimicrobial agents. From these studies, and others, it is clear that the exopolysaccharide matrix (or other components of biofilms) does not form an impenetrable barrier to the diffusion of antimicrobial agents, and other mechanisms must be in place to promote biofilm cell survival. However, for certain compounds, the exopolysaccharide matrix does represent an initial barrier that can delay penetration of the antimicrobial agent. The experiments described above strongly suggest that multiple mechanisms are required for overall antimicrobial resistance.

4.4 Slow growth and the stress response:

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antibiotics. Slow growth of the bacteria has been observed in mature biofilms. Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents.

5. Conclusion

Biofilms can be found on rocks and pebbles at the bottom of most streams or rivers and often form on the surface of stagnant pools of water. In fact, biofilms are important components of food chains in rivers and streams and are grazed by the aquatic invertebrates upon which many fish feed. Biofilms can grow in the most extreme environments: from, for example, the extremely hot, briny waters of hot springs ranging from very acidic to very alkaline, to frozen glaciers. In the human environment, biofilms can grow in showers very easily since they provide a moist and warm environment for the biofilm to thrive. It can form inside water and sewage pipes and cause clogging and corrosion

also on floors and counters can make sanitation difficult in food preparation areas. Biofilms in cooling- or heating-water systems are known to reduce heat transfer. Biofilms in marine engineering systems, such as pipelines of the offshore oil and gas industry, can lead to substantial corrosion problems. Corrosion is mainly due to abiotic factors; however, at least 20% of corrosion is caused by microorganisms that are attached to the metal subsurface (i.e., microbially influenced corrosion). Bacterial adhesion to boat hulls serves as the foundation for biofouling of seagoing vessels. Once a film of bacteria forms, it is easier for other marine organisms such as barnacles to attach. Such fouling can reduce maximum vessel speed by up to 20%, prolonging voyages and consuming fuel. Time in dry dock for refitting and repainting reduces the productivity of shipping assets, and the useful life of ships is also reduced due to corrosion and mechanical removal (scraping) of marine organisms from ships' hulls. Biofilms can also be harnessed for constructive purposes. Biofilms are found on the surface of and inside plants. They can either contribute to crop disease or, as in the case of nitrogen-fixing Rhizobium on roots, exist symbiotically with the plant. Examples of crop diseases related to biofilms include Citrus Canker, Pierce's Disease of grapes, and Bacterial Spot of plants such as peppers and tomatoes. Nowadays biofilms are used in microbial fuel cells (MFCs) to generate electricity from a variety of starting materials, including complex organic waste and renewable biomass.

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