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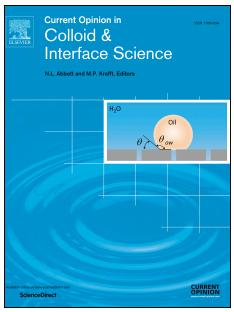

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Wetting/Spreading on Porous Media and on Deformable, Soluble Structured Substrates as a Model System for Studying the Effect of Morphology on Biofilms Wetting and for Assessing Anti-Biofilm Methods

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Abstract

Biofilm is a layer of syntrophic microorganisms stick to each other and to the surface. The importance of biofilms is enormous in various industrial applications and human everyday life. The effects of biofilm could be either positive or negative. Positive effects are encountered in industrial processes, bioremediation, and wastewater treatment. Negative effects are more common with the marine industry being one of the sectors which confronts severe corrosion problems caused by biofouling on the surfaces of equipment and infrastructures. In space industry, microbial contamination and biofouling adversely affect both crew health and mission-related equipment, the latter including hardware, water systems, piping, and electrical tools. The capacity of biofilms to grow in space environment was confirmed already in 1991. One of the most important surface properties of biofilms is wettability which dictates not only how a liquid spreads over the uneven external surface of biofilms but also how it penetrates into their porous and morphologically complex structure. To investigate wetting and spreading onto biofilms, model materials are often employed to simulate different morphological and functional features of biofilms in a controlled way, e.g., soft, deformable, soluble, structured, porous materials. Here we review recent advances in wetting and spreading on porous and soft deformable surface together with biofilms wetting properties and its importance in space industry. We conclude with a discussion of the main directions for future research efforts regarding biofilm wetting.

Contents

1. Introduction

Biofilms represent the most widely diffused and successful microbial way of life. The ability of bacteria to produce complex biofilm matrix, known as extracellular polymeric matrix (EPS), promotes colonization of biotic and abiotic surfaces, inducing stability in the growth environment and resistance against antibiotic and stress conditions [1].

Bacterial Biofilms, in various aspects, can be beneficial for nature and humankind as certain plants employ a coat of harmless biofilms i.e. these produced by *Bacillus subtilis* to protect themselves from pathogenic microorganisms [2, 3]. *Microalgal/cyanobacterial* biofilms are used in industrial processes and bioremediation [4-6], wastewater treatment in photobioreactors [7-9], and non-toxic leaching of copper from ore which rely on bacterial biofilms [8, 9]. Biofuels such as bioethanol can be produced through bioprocessing associated with biofilm as an energy efficient option without secondary pollution. *C. thermocellum* biofilm and Polymicrobial biofilms of *Bacillus subtilis* and *Staphylococcus aureus* are the examples of the strains used in this field [10]. Food industry can benefit from biofilms as biofilms of a probiotic bacteria, *Lactobacillus plantarum*, grown on nanofiber membranes are utilized as a starter culture for producing fermented milk [11]. Although biofilms are certainly actuating many industries, there are frequent cases where their presence and development might result in severe damages. In most industrial and medical settings, bacterial biofilms have a negative impact on the function of processes and devices [12]. Bacterial biofilms are the cause of almost 80% of the recurrent and chronic microbial infections which happen in human body. They can also be a source for inflammation when they grow on the medical device surfaces like implants. Microbial contamination and subsequent formation of biofilms on surgical implants frequently cause chronic infections that are difficult to eradicate. The risk of the infection depends on the type of the medical device, its invasiveness level, the site of insertion in human body, and the time during which it is applied into the anatomical site. When there is no external device, host immune defenses clear the tissue contaminations spontaneously. But when a foreign body, such as an implant is inserted into the target sites of human body, a local tissue response is triggered. This response alters the immune defense and creates a *locus minoris resistentiae*. This causes a vulnerability toward the bacterial attacks. Biofilms, being resistant to most of antimicrobial agents, spontaneous cure does never occur, and currently the available treatment for biofilm-related infections consists in the administration of conventional antibiotics at high doses for a long-term period. *Staphylococcus aureus* is one of the major implant-infecting bacteria. This strain shows a high rate of antibiotic resistance, just like *Staphylococcus epidermidis*, *Streptococcus* spp. and *Enterococcus* spp., which are also the examples of bacterial strains causing orthopedic infections [13, 14] and, are responsible for diseases which are difficult to fight [15, 16]. In case of Staphylococcal biofilms, they can be eliminated by rifampin combination therapy, and Gram-negative biofilms by fluoroquinolones but the treatment duration is 3 (hip prosthesis) and 6 (knee prosthesis) months, very often leading to implant exchange [17, 18].

Marine industry is one of the sectors which encounters severe corrosion caused by biofouling on the surfaces of equipment and infrastructures [19]. Colonization in marine biofouling can be performed by various organisms such as bacteria, diatoms, spores of macroalgae, protozoa, and larvae of macrofoulers. More importantly, with the growth of international trade in recent decades and especially of transoceanic maritime transport, littoral states have been confronted with ecological problems of a new order related to the contribution of living organisms foreign to the local environment [20, 21]. In aquatic and coastal environments, invasive species, such as the

bacterium *Vibrio anquillarum*, have been recognized as one of the serious threats to global biodiversity, and identified as one of the four greatest risks to the oceans with land-based sources of marine pollution, over-exploitation of living marine resources, and alteration or physical destruction of marine habitats [22, 23].

In space industry, microbial contamination and biofouling adversely affect both crew health and mission-related equipment including hardware, water systems, piping, and electrical tools [24, 25]. Onboard the International Space Station (ISS) biofilm formation [26] and consequently microbial contamination continues to pose mission risks, to crew wellbeing as the opportunistic pathogens in water systems and crew cabin present a serious health threat [27-30]. On the other hand, formation of biofilms on mechanical systems can seriously challenge the hardware reliability as they can also cause biofouling and material degradation, which can lead to system failure during long term missions [31-33]. Especially, that with increasing spacecraft complexity, crew numbers, duration of missions, and multiple flights for each spacecraft, new challenges have arisen for long-term control of microbial contamination and biofilm development in systems reused mission-to-mission, particularly in the water storage/distribution systems [28]. The growth of biofilms was confirmed in water and waste line samples, already in June 1991, after the STS-40 mission. On the Space Shuttle Columbia, despite continuous addition of iodine, bacterial biofilms such as *Burkholderia cepacia, Basillus spp,* and *Sphingomonas paucimobilis* were found, during the standard servicing protocols. Moreover, onboard the ISS [34], analysis of water samples from potable water sources have been performed already before the arrival of the first permanent crew. The results showed that the predominant microbial isolates were Gram negative bacteria such as *Cupriavidus metallidurans*, *Sphingomonas paucimobilis*, *Methylobacterium fujisawaense*, and *Wauteria basiensis*. This demonstrates the potential problems with the extended use of closed-loop systems and current control mechanisms.

Consequently, an increasing interest of several scientific communities is put to biofilms formation, growth, their microbial behavior and finally, to the development of efficient methods to eradicate bespoke biomaterial [35, 36]. Typical biofilm control strategies either aim at preventing bacterial attachment and thus biofilm formation, chemically inactivating the bacteria within the biofilm [37-39] or removing the whole biomaterial from surfaces by mechanical forces [12]. However, these traditional biofilm mitigation approaches are limited due to bacterial persistence and biocide resistance. Genetic modification of bacteria could represent a further possible strategy for fighting biofilm development. The modification of genes involved in biofilm formation and their development may have positive effects on these processes. Gene products with a negative effect can also be considered an excellent target to inhibit events needed for biofilm formation. The negative function of yeast-form cell wall protein 1 (Ywp1) in the adherence step might represent a positive function in biofilm dispersal and desegregation [40]. A different strategy to counteract biofilms development consists in the inhibition of genes that regulate key factors for biofilm production. In *Salmonella Typhimurium* the activation of the Rcs phosphorylation pathway results in the inhibition of the expression of genes encoding surface adhesins thus leading to the inhibition of biofilm formation [41].

As the biofilm covers the surface of a material a new surface with new properties is created. The wetting properties of such a newly formed surface are important in both exploiting the advantages of biofilms and preventing any detrimental consequences of their unfavorable effects. Surface parameters and wettability of biofilms are gaining increasing attention especially now that among the emerging technologies for combating biofilms, new surface coatings show promise for

preventing biofilm formation [42]. This approach aims to interrupt the critical initial step of biofilm formation (cell attachment) through surface modification.

The development of materials capable of preventing or inhibiting bacterial attachment on medical devices might represent an important alternative to the use of biocide substances. Several different approaches that involve physical and chemical surface modification have been proposed. The engineered surfaces can be coated with molecules capable of inhibiting bacterial adhesion or with active antimicrobial agents. Moreover, surface treatment with natural disruptive agents and modification of surface topographical parameters should also be considered to disrupt the biofilm matrix [43]. Furthermore, the essential oils EOs from aromatic plants were screened for their ability to prevent biofilm formation and to disrupt preformed biofilms against clinical and *Methicillin Resistant Staphylococcus aureus* (MRSA) strains [44]. Finally, very recently, hydrolytic enzymes secreted by bacterial cells like dispersin B have been employed to degrade the components of the biofilm polymeric matrix of *S. epidermidis, Burkholderia cenocepacia, and Achromobacter xylosoxidans* leading to active dispersal of the biofilm with a reduction of the biomass [17].

Besides chemicals, also physical strategies have been addressed toward biofilm disruption; low cytotoxicity magnetic nanoparticles (MNPs) in combination with magnetic fields were shown to provide a deep penetration into the biofilm damaging the biofilm matrix and causing detachment [45]. Finally, modification of surface topographical parameters is able to reduce the attachment of microorganisms on materials for long time providing a local and well-characterized distribution of topographical patterns [46].

Biofilm resistant coatings can eliminate or reduce the need for disinfectants, reduce the environmental marine pollution and, avoid the development of biocide resistant “superbugs,” thus offering distinguishable advantages for biofilm prevention during long duration missions. The microscopic organisms tend to move toward the material surfaces and form aggregations on these nutrient-rich surfaces because of the concentration gradient of nutrients. As this bacterial movement is stimulated by a directional exogenous factor, it is called taxis. The taxis caused by the nutrients is chemotaxis. The adsorption of chemical materials and the attachment of the microorganisms forms a film onto the surface. In comparison with the substrate, this new thin layer has different surface characteristics, such as surface charge, hydrophilicity/hydrophobicity, surface tension, surface free energy, roughness, and wettability. This system is a non-ideal surface containing pores and microgrooves and possessing deformable structure. It means that their interfacial characteristics such as wettability cannot be evaluated by equations and models used for ideal flat solid surfaces [47-49]. Therefore, wetting properties and/or spreading characteristics of biofilms along with their adsorption capabilities and adhesive parameters on porous media are noteworthy to be studied as a matter of priority.

In order to optimize the design of the future space exploration vehicle for long term missions, new technologies, in which the superfacial and wetting properties have to be considered, are needed to control the habitat microbial environment over multiple years.

2. Biofilm Structure

Bacteria generally grow in one of two ways: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface or within the confinement of a biofilm. A biofilm consists of a

microbial community sheltered in matrix of extracellular polymeric substances, EPS, that include proteins, polysaccharides and, surface-associated microorganisms such as bacteria, fungi, algae, protozoa, extracellular DNA [50, 51]. Together with EPS, pili, flagella and other adhesive fibres secreted by the microorganisms, act as a stabilizing scaffold for the three-dimensional biofilm structure. Flagella and pili are the structures on the outer surfaces of bacteria. These organelles enable the bacteria to interact with their environment. There is a potential influence of bacterial motility in contaminated liquids, and their accumulation on specific regions of the surface, on biofilm formation and structure, even if these aspects are still not fully investigated. Nutrients, in the matrix, are trapped for metabolic utilizations by the resident bacteria and water is efficiently retained through H-bond interactions with hydrophilic polysaccharides [52, 53]. Enzymes secreted by the bacteria modify EPS composition in response to changes in nutrient availability, thereby tailoring biofilm architecture to the specific environment [38]. Thus, the structural components of the matrix give rise to a highly hydrated, robust structure with high tensile strength that keeps bacteria in close proximity, enabling cell-to-cell interactions and DNA exchange, while protecting the biomass from environmental stresses, creating an inhomogeneous, porous thin layer, that represents a new surface with newfound properties.

2.1. Biofilm formation

Biofilm formation requires five stages: (i) reversible attachment of the bacteria to the substrate followed by (ii) irreversible attachment of cells to a solid substrate, the key step in biofilm formation, (iii) first maturation though which microcolonies grow and become thick, (iv) second maturation, in which microcolonies get the maximum size and, (v) detachment [54]. Colonization is the first action in this process to overcome repulsive forces between bacteria and the surface allowing the initial contact and translocation. Mechanisms governing bacterial adhesion at the single-cell level are different, and depend on cell type, surface physic and chemistry, and the liquid environment. It is not possible to draw a general description about how adhesion is achieved at the single-cell level, however a wide discussion of the phenomena, including an analysis of the physical forces experienced by a cell before reaching the surface have recently been discussed by Berne et al [55]. Once single cells are attached to the surface they start to multiply and form communities. Some other bacterial cells interact with surface, divide, and leave. A multigeneration memory of this mechanism allow future generations to return to the surfaces and progressively better adapting to surface sensing and attachment [56]. To protect and strength colony adhesion to the surface an extracellular matrix is formed.

A multitude of proteins play essential roles at different stage of this process. Some proteins contribute to biofilm accumulation while others are involved into the mediation of primary attachment to surfaces or the matrix development. Each stage of the biofilm formation process depends on the microbial genera, species, characteristics of the attachment surface, environmental conditions, external stress and physiological status of the microorganism [57]. Bacteria involved into the biofilm matrix are more tolerant to antibiotics than planktonic cells. This antibiotic resistance can be related to the increased transmission of resistance markers, efflux pumps, physical protection and acquired resistance. Biofilms have also dynamic structural properties and rapid alterations in their gene expression lead to modification of their surface antigens [58].

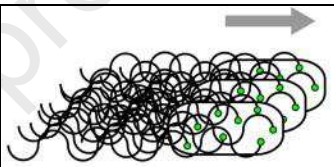
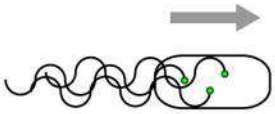
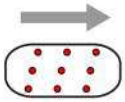
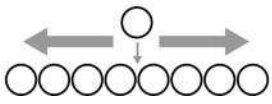
2.2. Bacterial motility

Biofilms are usually investigated in static conditions that, however, are very far from reality as in the vast majority of cases, biofilms form under fluid flow with the flow playing a significant role in the production, composition and architecture of the biofilm [34, 59]. The fluid flow drives bacteria motility favouring surface colonization.

Bacterial mobility is enabled by two different types of structures, flagella, fimbriae, and pili. Flagella are a lash-like appendage that protrude from the cell body, are made of three basic parts: a filament, a hook, and a basal body, cells can have one or more flagella. Fimbriae and pili are thin, protein tubes originating from the cytoplasmic membrane that is rapidly polymerized and depolymerized assembling protein subunits called pilin [60]. Both are able to stick bacteria to surfaces, but pili are typically longer and fewer in number than fimbriae. They are found in virtually all Gram-negative bacteria but not in many Gram-positive bacteria. At the end of tube is the adhesive tip structure based on glycoprotein or glycolipid receptors. These structures are necessary for the movement towards surfaces, allowing microcolonies formation and initial bacterial adhesion [33].

Different motility mechanisms can be identified [61], a brief summary is reported in Table 1.

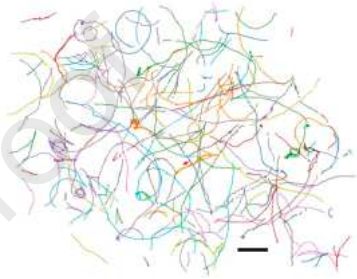
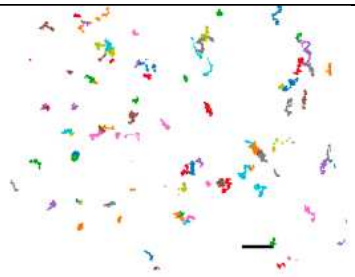
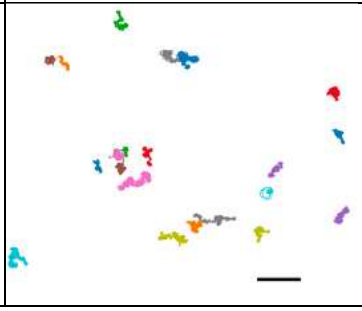
Table 1: Bacterial motility mechanisms [61].

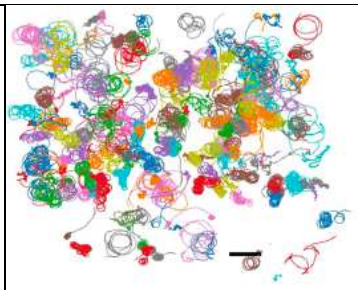
Swarming motility (flagella)	Defined as a rapid multicellular bacterial surface movement powered by rotating flagella.	
Swimming motility (flagella)	Movement powered by rotating flagella but takes place as individual cells moving in liquid environments.	
Twitching motility (pilius retraction)	Surface motility powered by the cyclic extension and retraction of type IV pili that confers slow cell movement often with a jerky or "twitchy" appearance	
Gliding motility (focal adhesion complexes)	A catch-all definition for active surface movement that occurs along the long axis of the cell without the aid of either flagella or pili.	
Sliding motility (spreading by growth)	Passive form of surface spreading that does not require an active motor, but instead relies on surfactants to reduce surface tension enabling the colony to spread away from the origin driven by the outward pressure of cell growth.	

When there is a cell transition from swimming to swarming, the number of flagella on the cell surface increases. Organisms with alternative flagellar systems become hyperflagellated in the

297 transition from the single polar to multiple peritrichous flagella. Chemotaxis and surface sensing can
298 influence directionality and motility mechanisms.
299 Analysis of trajectories of P. aeruginosa PA01 (monotrichous bacteria, propelled by a single
300 flagellum located at the pole at one end of the cell body) in an oil/water emulsion [62] evidenced
301 four distinct characteristic motions, summarized in Table 2:
302

303 Table 2: Description of different bacteria trajectories at the Oil-Water interface (from fig 2 in [62]). Scale bars
304 are 20 µm.

Motility type	Population frequency	Description		Trajectories
Interfacial visitors	10−20%	Are not adhered but swim toward and away from the interface, changing their heights by several micrometers.		
Brownian Di□usive Bacteria	30%	Are similar to inert passive colloid trapped at the interface. The bacteria are probably in a sessile, inert state or are trapped in a configuration that denies the molecular motor access to ions that fuel its rotation, for example, by immersion of the flagella in the oil phase.		
Pirouettes	(rare, ~ 5%)	Rotate quickly in nearly fixed positions.		

Curly Paths	~40%	Swim in curly paths more than any other mode of motion; the trajectories are quite stable except in the event that they collide with other bacteria and become trapped in a cluster.		

By using advanced microscopy techniques, such as dual-view light-sheet microscopy, it is possible to monitor spatial trajectories of individual cells and the collective motion that lead the biofilm expansion. Trajectories of early born cells (0-7 hours) are more trapped at the substrate with respect to cells born later (12-15 hours) [63].

In the initial phase (0-5 hours), the biofilm grew predominantly in the lateral plane and cells shown a Brownian and random walk. As the biofilm develops (5-10 hours), individual cells shown persistent and straight trajectories, which dominate the bulk of the biofilm at the later stage (10-15 hours). Biofilm expansion is driven by cell division, extracellular matrix secretion, and osmotic swelling. The Brownian-to-ballistic transition of cell motion coincided with the transition from predominantly lateral biofilm expansion to accelerated vertical expansion, a transition from 2D to 3D.

2.3. Spreading of the bacteria laden droplets on solid substrates

Secchi et all. developed a mathematical model of bacteria swimming in flow using microfluidic strategy and *Pseudomonas aeruginosa* and *Escherichia coli* as model and provided a new tool to predict the location and magnitude of bacterial attachment to surfaces [64]. Hydrophobic coating [65, 66] can prevent biofilm formation on different surfaces, affecting wettability and surface properties. Other studies investigated the possibility to inhibit contamination of medical implants by treating titanium surfaces by radio-frequency cold plasma [67].

Inhibition of bacterial mobility and/or swimming decrease biofilm formation in many pathogenic strains. Inactivation of the PA5001 gene in *P. aeruginosa* generated a nonmobile strain resulting in the alteration and disruption of biofilm matrix [68]. A similar effect was observed in *P. aeruginosa* after treatment with plant-derived phenolic compounds; the swarming motility and consequently biofilm production were reduced by about 50% [69]. In *Enterococcus faecalis*, CLSM and SEM analysis demonstrated that treatment with phenyllactic acid (PLA) affects cell motility and reduces EPS production inhibiting bacterial adherence and biofilm formation [70]. Several antimicrobial peptides were demonstrated to affect biofilm formation at different stages and through different mechanisms of action. Human cathelicidin LL-37 peptide inhibits *P. aeruginosa* biofilm formation by downregulating genes related to the QS system, decreasing the ability of bacterial cells to attach on surfaces and stimulating twitching motility mediated by type IV pili. The CRAMP antimicrobial peptide is able to inhibit fungal biofilm formation and a CRAMP short fragment, the AS10 peptide, was shown to inhibit biofilm growth of *P. aeruginosa*, *E. coli*, and *Candida albicans* [71]. A novel synthetic cationic peptide, defined as 1037, is able to affect biofilm formation by downregulating several genes related to flagella decreasing swimming motility in PA14, PAO1, and Burkholderia cenocepacia, and suppressing the expression of a variety of genes involved in biofilm formation [72]. AMPs can also cause disruption of the biofilm matrix. The hepcidin 20 peptide can reduce the

mass of the extracellular matrix altering the *S. epidermidis* biofilm architecture by targeting polysaccharide intercellular adhesin (PIA) [54].

Bacteria motility can induce formation of aggregates and affect interfacial properties in the case of multiphase systems (droplets). Motile bacteria can aggregate in polymer-rich environment via polymer-induced depletion forces. In the presence of non-adsorbing polymers such as polyethylene glycol (PEG), bacteria aggregate through depletion interactions, which occur when two bacteria approach each other and reach a depletion zone where the polymer is excluded from the space between them: this force is expressed as an osmotic pressure difference generated from the variation in polymer concentration between the depletion zone and the bulk solution. For non-motile bacteria, the only driving forces for aggregation are polymer-induced depletion forces. For motile bacteria, motility forces and depletion forces competition determine a steady-state aggregation behaviour at sufficient polymer concentrations and long-time scales. Porter et al [73] by measuring size distribution of bacterial aggregates using confocal microscopy, showed that motility influences the polymer-induced depletion aggregation of bacteria at short time scales (10 min). In dilute polymer concentrations, aggregation of nonmotile bacteria is observed but no aggregation of motile bacteria because the depletion forces are simply not strong enough to compete with the swimming forces. In the semi-dilute regime, in a viscous environment, when a critical PEG concentration threshold is reached the aggregation starts also for motile bacteria.
Bacterial motility can heavily affect interfacial properties also in the case when bacteria are present in a droplet of liquid wetting a surface.
A water drop can slide on a tilted plane of agar gel when the driving force (gravitational) overcomes the capillary pinning force, i.e. when the value of the Bond number (Eq. 1) reaches a critical value:

$$Bo = \frac{\rho V\, g sin(\alpha)}{V^{1/3}\gamma}, \qquad \text{Eq. 1}$$

where ρV and $V^{1/3}$ are the drop mass and typical width, $gsin(\alpha)$ is the effective gravity, and γ is the surface tension.

Bacteria can unpin such droplets, leading in practice to the collective 'surfing' of the entire colony. Hennes et al [74] observed the sliding of bacteria-laden droplets with an initial Bond number of Bo=$3\cdot 10^{-3}$, whereas water drops only start sliding for Bond numbers larger than Bo≈0.25.
Bacteria influenced the Bond-number of the drop in following ways:

1. Pump water from the environment can increase drop volume
2. Surfactant secretion can lower the surface tension (*B. subtilis secretes surfactin*), strongly enhancing the wettability of the agar gel.

In the case of E. Coli moving a sub-millimetric emulsion drop [75], each motile bacteria can induce force of magnitude, f, (Eq. 2)

$$f \sim \eta l v_0, \qquad \text{Eq. 2}$$

where v_0 and l are the characteristic speed and size of the bacterium, and η the viscosity of the surrounding fluid. The energy required to create a "bump" of size comparable to the bacterial body in the drop surface ($\sim \gamma l^2$) is lower than the interfacial tension [73], while the energy that a bacterium spends by swimming the same distance is (Eq. 3)

$$fl \sim \eta l^2 v_0 \qquad \text{Eq. 3}$$

The ratio between these two energies is of the order of the capillary number (Eq.4),

$$Ca = \frac{\eta v_0}{\gamma} \qquad \text{Eq. 4}$$

For a typical water-oil interface in the presence of surfactants, $\gamma \sim 1$ mN/m and $Ca \sim 10^{-5}$.

As a result, bacteria swimming near a typical water/oil interface feel a rigid boundary and thus behave like swimming near a solid wall rather than a free surface; they interact hydrodynamically and accumulate at the interface. This accumulation near the drop interface can enhance the interaction of the bacterial flows in the drop (Figure 1a) and the fluid surrounding the drop. It is shown that the drop movement and its direction is determined by the bacteria that move near the substrate, causing the drop to roll over the substrate. The turbulent-like motion of the bacterial bath constantly changes the direction and speed of the bacteria that swim near the bottom of the drop. This explains both, the persistent movement of the droplets at short times and their random motion at long times.

2.4. Coffee ring effect

Swimming cells in a drop do not distribute randomly. Particles in an evaporating droplet accumulate at the interface and typically leave a ring-like pattern on the underlying substrate after complete evaporation, a phenomena commonly known as “coffee ring effect” When bacteria produce surfactants, the pattern of coffee ring deposition does not appear [76]. The presence of gradients of nutrients (such as sugar) induce the bacteria to move toward the nutrient site with resulting convective flows (Figure 1b). This may be attributed to the fact that bacterial chemotaxis near the base dominates over the internal fluid flow, while away from the sugar crystal, chemotaxis is relatively weaker. Chemotaxis can hence influence live bacteria deposition and motion in a drop.
This non-random distribution of bacterial, and their accumulation in specific areas of the surface can be expected to influence surface contamination and biofilm formation, for example by inducing surface tension gradients. A clear study about direct connection between bacterial motility inside a contaminated droplet and spreading of biofilm is not yet available to our knowledge, but we believe that investigation of wetting of bacterial-laden droplets on clear surfaces could represent a promising approach to study surface contamination by droplets.

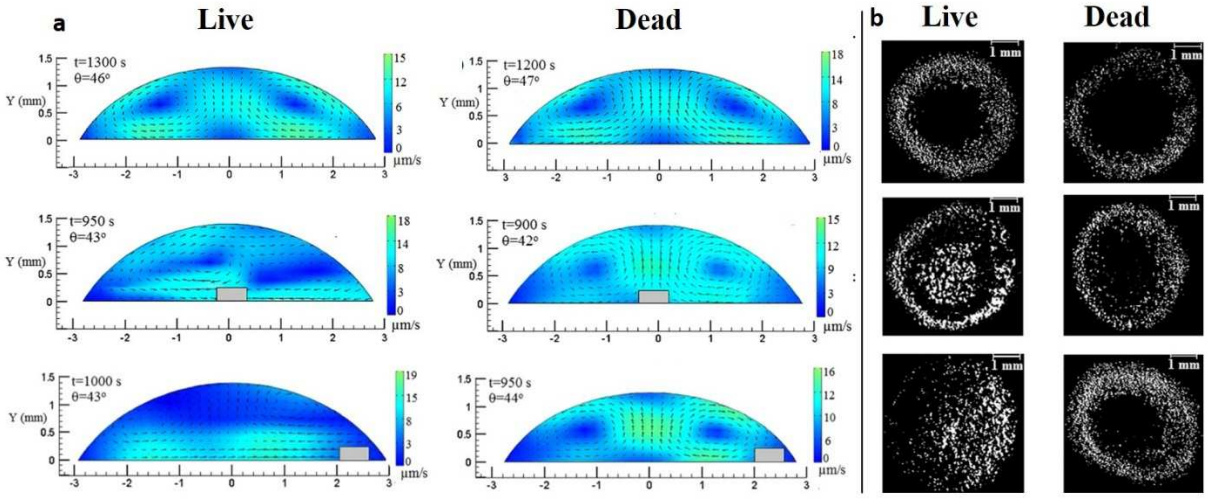


Figure 1 Fluid flow measured inside an evaporating droplet using the PIV technique [76] show that the presence of a chemoattractant can influence the spatial distribution of bacteria: a) velocity vectors are superimposed over velocity

contour, during droplet evaporation comparing the case of live (swimming) and dead bacteria; b) grey images compare the deposition pattern of live and dead cells after complete drying of the droplet, top image are in the absence of sugar, in the case of middle images sugar was deposited at the centre of the droplet (grey rectangle), in the bottom line sugar is on the right side of the droplet.

Inclusion of bacteria in drops can be controlled using microfluidic concepts to create monodisperse double and triple emulsion drops that serve as 3D microenvironments for the containment and growth of bacterial biofilms. B. subtilis [77] was encapsulated in an aqueous suspension of planktonic bacterial cells to create w/o/w double emulsion drops with an outer diameter of ≈164 ± 4 µm. Within 24 h, these planktonic cells multiply and differentiate into matrix-forming cells at the inner interface of these microscopic drops, forming 3D spherical biofilms on the inside of the oil shells. The inner water–oil (w/o) interface was stabilized with a silicone surfactant, which is a known film-former, and provides a substrate upon which the biofilm readily adheres.
An overall decrease in drop size is observed as the biofilm grows. The calculated inner water volume decreases by 45% over the first 12 h and then remains constant. This corresponds to the peak in matrix-production. Thus, this decrease in volume can be attributed to nutrient depletion, which creates an osmotically driven water flux from the inner aqueous phase to the outer continuous phase.

3. Wetting/Spreading on Porous media

3.1. Biofilm topography

As already mentioned, surfaces of materials in different environments will inevitably be coated by carbon compounds as nutrients. At first, proteins are adsorbed onto the surfaces and this is followed by carbohydrate adsorption. A formed layer of nutrients is called conditioning film. As the microscopic organisms tend to move toward the material surfaces, they form aggregations on these nutrient-rich surfaces due to the concentration gradient of nutrients. Thus, the adsorption of chemical materials and the attachment of the microorganisms change the characteristics of the surface, such as surface charge, hydrophilicity/hydrophobicity, surface tension, surface free energy, roughness, and wettability [47].

Biofilm can be considered as a porous, thin layer in which the fraction of void space is a characteristic parameter for modelling the structure of the biofilm. Imaging techniques, such as confocal laser scanning microscopy (CLSM), magnetic resonance microscopy, multiphoton-excitation laser scanning microscopy (MPLSM), and near-infrared optical coherence tomography (OCT) have been applied to indicate and analyze the morphological parameters including porosity, pore size distributions, and roughness [78]. Figure 22 shows the two-dimensional and three-dimensional views of the biofilm morphology using OCT system [78, 79]. The pore radius in the structure of the biofilms is of micron (µm) order. Based on the experimental observations about this structure, pore-scale models are utilized for biofilm formation. These models consider the biofilm as a porous medium [80].

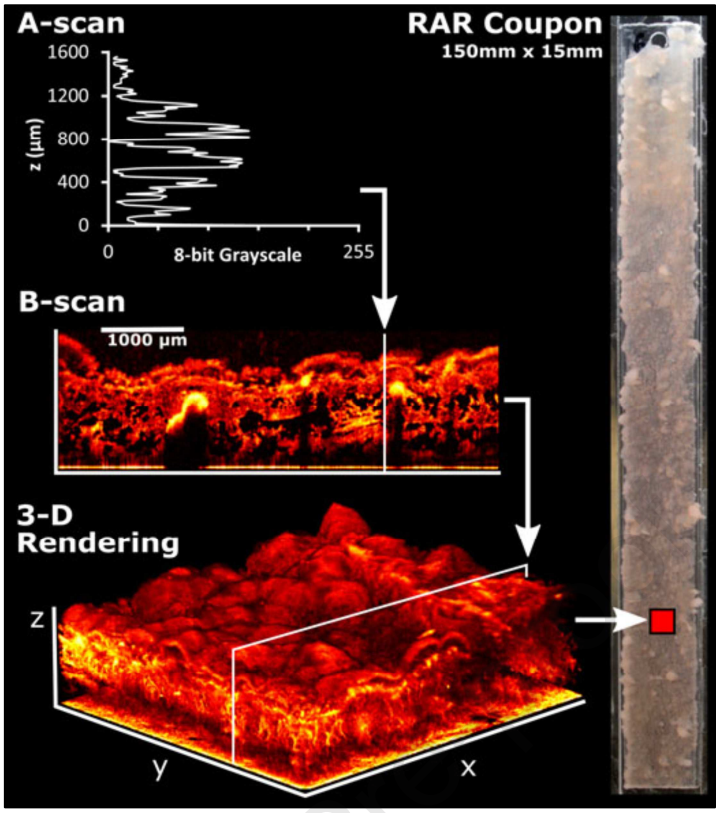


Figure 2 Two- and three-dimensional images of the biofilm of ammonia-oxidising bacteria (AOB) on polycarbonate coupons in the rotating annular reactor (RAR) using optical coherence tomography (OCT). To produce the 3-D rendering image of the morphology, multiple adjacent A-scans as the vertical one-dimensional profiles with grayscale intensity are collected and assembled to generate B-scan as the two-dimensional images. Then B-scans are used to render three-dimensional images [79].

Beside the structure of the biofilm, its mechanical properties influenced by the morphology are also of a great importance for predicting the behavior of biofilms, their control and even removal [81]. To ascertain these mechanical characteristics, both experimental measurements and modelling methods are used together. OCT technique has demonstrated the two-dimensional deformation of the biofilms. This imaging method together with poroelastic fluid-structure interaction numerical computations result in developing a method for determining the elastic properties of the biofilm as a deformable structure [82]. Due to the porosity and elasticity of the structure, it is quite accurate to consider the biofilm as a porous medium and/or deformable substrate when it is in contact with other materials. This hypothesis about the biofilms is employed to investigate their wetting properties as a significant interfacial characteristic when it comes to either the comprehensive range of applications or the necessity of removal of the biofilms.

3.2. Wetting of Biofilms as Porous Substrates

Wetting is an indicator of the behavior of a unique liquid on the surface. For biofilms, this indicator depends on surface topography. The concept of wetting can be defined by the contact angle (CA) which quantifies the wettability. Therefore, measuring the CA on the porous and rough surfaces of the biofilms determines its wettability. As it was mentioned before, the size of the pores

radius in the biofilms is of µm order. This means that biofilms have micropatterned surfaces on which two states of wetting can be distinguished: (1) the Wenzel state, and (2) the Cassie-Baxter state [83]. In the Wenzel state, the liquid fully wets the porous structure. Based on this assumption, the apparent CA, θ_{ap}, is calculated by Wenzel equation as below:

$$\cos\theta_{ap} = r\cos\theta_{eq} \qquad \text{Eq. 5}$$

where r and θ_{eq} are the roughness ratio and Young's angle, respectively [84]. The equation for Young's angle is:

$$\gamma\cos\theta_{eq} = \gamma_{SV} - \gamma_{SL} \qquad \text{Eq. 6}$$

where γ, γ_{SV}, and γ_{SL} are the representative of liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively. In the Cassie-Baxter state, the wetting is partial so that the liquid droplet sits on the top of the protrusions of the rough surface. The proposed equation by Cassie-Baxter is:

$$\cos\theta_{ap} = f'\cos\theta_{eq} + f' - 1 \qquad \text{Eq. 7}$$

where f' is the area fraction of the liquid-vapor interface blocked by the rough structure [85].

Topographical characterizations can be conducted by a profilometer. Using light profilometry images obtained by this system, the developed interfacial area ratio is calculated. In a relevant study, the behavior of water droplet on different biofilm surfaces was investigated [12]. Three distinct states were demonstrated: hydrophilic, hydrophobic rose-like, and hydrophobic lotus-like biofilms. On rose petal-like surfaces, the water can penetrate into the microscopic pores of the underlying surface which results in notable contact angle hysteresis. In this case, called impregnated Cassie state, the droplets remains attached when the surface is tilted. The impregnated Cassie state is a state between Wenzel and Cassie-Baxter states. In case of lotus-like biofilms, when the surface is turned upside down or tilted, the droplet rolls off. Lotus-like behavior is the representative of the Cassie-Baxter state. Figure 3 shows the wetting behavior of different biofilms exposed to the water droplet [12].

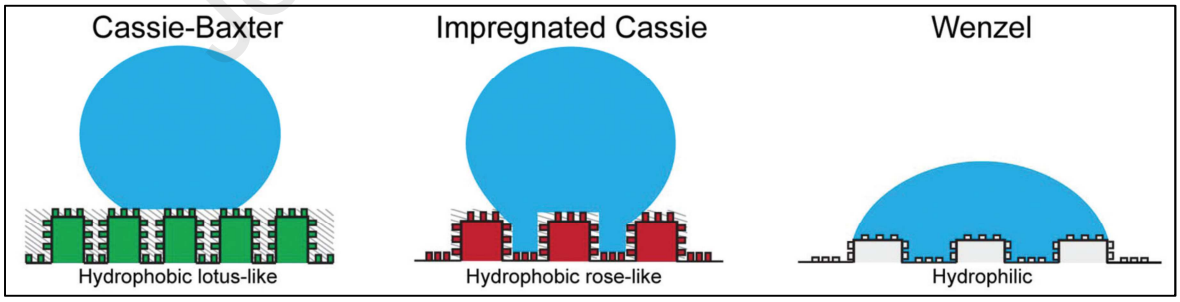


Figure 3 Wetting behaviour of the rough surfaces of different biofilms in contact with a water droplet [12].

The importance of the wetting concept of biofilms can be divided into three areas: (a) to control the behavior of biofilm during its interactions with other materials such as a reactant liquid which flows in the reactor during its operation, (b) to predict the interactions between the biofilm surface and chemical agents used for its removal, and (c) to modify the different surfaces to impart antibiofilm characteristics. The last area, which is related to the wetting phenomena for biofilms, is different from the first two areas. In this case, wetting properties of a surface is manipulated by physical and/or chemical methods so that the final surface exhibits antibiofilm or antibiofouling

features. To clarify, changing a surface from a hydrophilic character to a hydrophobic one shifts the adhesion of microorganisms onto this surface.

3.3. Wetting of Soft / Deformable Substrates

Alike to porous medium, soft / deformable substrates can be proposed as the second model for investigation of wetting properties of biofilms. Wetting on soft substrates is not captured by the laws dominating rigid wetting phenomena.

The structure of a soft biofilm is deformed by the deposition of a droplet on it. This happens because of the surface tension and Laplace pressure, ΔP, of the droplet. According to Young's equation, Eq. 66, there is an in-plain balance between the three interfacial tensions at the three-phase contact line (Figure 44, a). The vertical component of liquid-vapor surface tension, $\gamma \sin\theta_{eq}$, remains unbalanced. So, a vertical net force is exerted to the solid surface at the three-phase contact line. In addition, Laplace pressure is applied to the liquid-solid interface (Figure 44, b). This pressure is inversely proportional to the curvature of the droplet. Consequently, a wetting ridge, δ, with a length scale of the order of elastocapillary length, L_{ec}, is formed at the three-phase contact line [86]. This ridge considerably changes the macroscopic spreading dynamics [87].

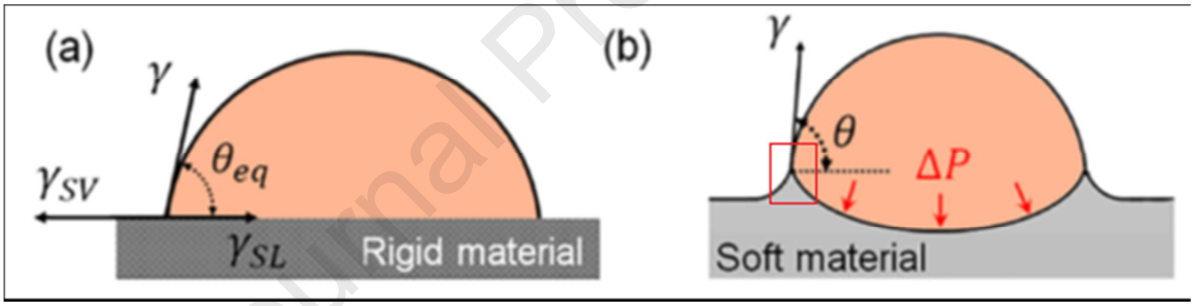


Figure 4 A liquid droplet deposited on the (a) rigid and (b) soft material. The red square in (b) is the wetting ridge [86, 88].

The elastic / shear modulus, G, for biofilms has been predicted to be between 0.7 and 7 kPa [82] which, according to Eq. 88, leads to a wetting ridge, δ, of sub-millimeter scale. Therefore, it can affect the wettability [86, 89].

$$\delta \sim L_{ec} = \frac{\gamma}{G} \qquad \text{Eq. 8}$$

Both static and dynamic wetting properties of biofilms are affected by their deformation when they are in contact with the liquid droplets. In static wetting, deformations rebalance the interfacial tensions and modify the contact angle and contact angle hysteresis. In case of dynamic wetting, the wetting ridge moves with the contact line. This movement results in additional energy dissipation and influences dynamic wetting [86].

In addition to the force balance near the three-phase contact line, there are other characteristics and features which must be noted in this case. The dynamic solid surface tension the microstructure of the underlying polymer which is a combination of EPS and microorganisms in case of biofilms, boundary conditions, moving contact lines, the mechanisms of dissipation inside the substrate, and

the consequent macroscopic movement of the droplets are the other factors which must be revisited [87].

4. Wetting of Biofilm covered surfaces

The wetting of biofilm covered surfaces is a complex phenomenon. Considering a droplet in the size scale of millimeters as it is of practical interest in many applications. Most of the droplet surface shape is still described by the classical Young-Laplace equation. However, in the region of the biofilm e.g., size order up to 100 μm, a new phenomenon appears. The first one refers to the partial adsorption of liquid to the biofilm. Its extent depends on the properties of the biofilm and of the liquid. The second phenomenon is the modification of the triple line location and of the contact angle distribution along this line. The former effect is similar to what is met in wetting of porous media, in particular of a thin and loose porous media, discussed in section 3. The latter effect is related to the wetting of structurally and chemically heterogeneous surfaces about which there is very extensive literature [90]. It is clear, that both, the structure (the term "topology" is also used) and the composition of the biofilm affect its wetting behaviour. A complete three-dimensional experimental knowledge of these quantities is out of the question by today means so by necessity modelling must be invoked to expand our understanding on biofilm formation and structure/composition. A discussion of the available modelling approaches of biofilm structure and composition follows since the biofilm modelling will be in the future an indispensable tool to understand its wetting and to correlate wetting properties to biofilm formation conditions.

Biofilm formation is a "nucleation"- “growth” process which explains the highly non-uniform structure arising. The "nucleation" step is actually the microbial deposition and attachment stage. The physical-chemistry of this step has been recently reviewed in detail by Carniello [91]. Some key approaches to modeling of the biofilm growth is described here. A basic classification separates morphological (i.e. 2 or 3 spatial dimensions) from non-morphological (i.e. 0 or 1 spatial dimension) models. The landmark work on biofilm growth is [92] which combines complete solution of flow field and nutrient concentration equations in the biofilm considering it as complex porous medium. As already mentioned, the biofilm composition is described as a combination of cells (at different states) and extracellular polymeric substance (EPS). Additional phenomena such as chemical mechanical stresses and quorum sensing are also taken into account. The biofilm shape evolution is determined by a cellular automata-like procedure. The detachment of biofilm pieces is also considered in the model. "Nucleation" is introduced by following trajectories of planktonic microbes. The transport properties in the biofilm are related to its local composition through an effective medium approach. The model is numerically solved by an in-house code. However, the required computational effort is too high for any practical use of it. A 2-D case simulation needed 5 days of computer time.

The computational effort is attempted to be reduced by ignoring stress effect and biofilm composition, introducing the concept of an effective viscosity to simulate the flow in the biofilm and implementing the code in a combination of Matlab, COMSOL Multiphysics and Java environments [93]. A simpler in-house cellular automata algorithm is implemented. The position of "nucleation" is randomly selected among the surfaces with local shear stress lower than a prescribed value. The above modification made possible the simulation of 3-D biofilm growth for several cases (simple in practical context since a single nutrient and a single microbe are considered) with the highly

localized character of colonies to be evident. The most sophisticated biofilm model today is the one presented in [94] that considers multiphasic hydrodynamic theory and takes into account interactions among various bacterial phenotypes, extracellular polymeric substance, quorum sensing (QS) molecules, solvent, and antibiotics. In the model, bacteria are classified into down-regulated QS, up-regulated QS, and non-QS cells based on their QS ability. The evolution of biofilm is determined by combining Cahn–Hilliard type equations for each substance. The model is capable to give 3-D results for the biofilm structure.

Another category of models sacrifices dimensionality to increase sophistication of film composition description. In this case the model is 1-D so it is completely continuum (no need for cellular automata). In addition, no flow in the biofilm has to be resolved. Such a model in [95] covers the possibility for simultaneous existence of several microbial types and several nutrients. It is specifically focused on the release of planktonic bacteria from biofilm to the bulk liquid. This process is different from the detachment since these bacteria are produced throughout the biofilm volume due to phenotypic change of the attached bacteria.

Finally, the last category refers to very abstractive 1-D models which are based on the diffusion-reaction equation of a single nutrient [96]. The difference from the previous case is that a series of simplifications (i.e. linearization of the reaction rate) brings the problem to a standard form in reaction-diffusion physics. A roughness elimination force is introduced through the notion of an artificial "surface tension" of the biofilm. A stability analysis of the model (assuming a deformed second dimension) is performed leading to phase diagrams for stable (flat) and unstable (rough) film growth. Obviously, this type of modeling is only of academic and not of practical merit.

From the above it can be inferred that the existing models are too simplified to use relevant information or too complex to be constructively used in the context of the wetting properties of biofilms. There is a need for reduced order models that have as state variable a finite set of descriptors determining the wetting behavior. In case of a wetted biofilm, the simplest set could be its average thickness, its EPS content and an integral roughness descriptor.

It appears that most research on wetting of biofilms focuses on the particular case of *Bacilus Subtilis* (BS), A *Gram-positive soil bacterium*, biofilms. These particular biofilms attract interest because they are non-wettable, not only with respect to water but for all liquids, including antimicrobial agents. Such omniphobic behaviour creates the need for fundamental analysis, in order to explain its origin, on one hand and, for practical methods to overcome microbial resistance to biocides on the other. The landmark work on the subject is reported by Epstein [97]. In that study, the contact angle created between droplets of several liquids and biofilms is measured through a simple goniometer. The main comparison is performed with respect to a Teflon surface. Although both biofilm and Teflon are non-wettable by pure water (with the contact angle to be higher for the biofilm), the contact angle on Teflon decreases linearly with the percentage of ethanol concentration in the liquid but the contact angle on biofilm remains constant up to 60% ethanol. Then it starts to decrease gradually and at 100% ethanol it reaches the contact angle of Teflon (highly wettable). The relevant figure has appeared extensively in literature [98]. It is also shown that a similar behaviour holds for isopropanol, methanol and acetone. Parameters like biofilm age, time of liquid exposure, repeated liquid contact appears to have no effect on biofilm-liquid repellency. Experiments using several mutants of *Bacilus Subtilis* (to assess chemical contributions) and epoxy resin replicas (to assess structural contributions) lead to the conclusion that the biofilm nonwetting properties arise from both the polysaccharide and protein components of the extracellular matrix and are a synergistic result of surface chemistry, multiscale surface roughness, and re-entrant topography. Additional biological

analysis focused on further explanation of the chemical contribution to liquid repellency in [99]. The conclusion is that it is conferred by a small concealed protein called *BslA*, which self-assembles into an organized lattice at an interface. In the biofilm, production of *BslA* is tightly regulated and the resultant protein is secreted into the extracellular environment where it forms a very effective communal barrier allowing the resident *Bacilus Subtilis* cells to shelter under the protection of a protein raincoat.

5. Conclusions

A microbial community sheltered in a matrix of extracellular polymeric substances called EPS, including polysaccharides, proteins, and extracellular DNA, create a layer of biofilm. Together with pili, flagella, other adhesive fibres, EPS act as a stabilizing scaffold for the three-dimensional biofilm structure which can be considered as a porous thin layer, and which due to nucleation process yields a highly non-uniform structure. Creating a new surface with newfound properties, is important in both, exploiting the advantages of biofilms in various applications and, preventing any detrimental consequences of their unfavourable effects. Their impact can be widely observed in the extended use of closed-loop systems and control mechanisms, affecting humankind safety or even life, especially in conditions where preventing any of detrimental consequences of their unfavourable effects is extremely difficult i.e. in microgravity conditions such as in the International Space Station or in Space Shuttles. There, the microbial contamination and biofouling events adversely pose mission risk, presenting a serious health threat to crew but also challenging reliability of the mission-related equipment.

This is why one of the most important aspects of biofilms research, which should not be overlooked, regarding biofilms prevention or/and control strategies, are their surface properties and wettability. This is especially true now that among the emerging technologies for combating biofilms, new surface coatings show promise for preventing biofilm formation.

The study of wetting properties, and surface interaction of droplets/bubbles can represent a useful and innovative tool to investigate the phenomena of surface contamination, including the prevention of biofilm formation, and optimization of its removal. Two different aspects should be considered, both deserving further investigation in our opinion. On one side the study of the interaction of bacterial-laden droplets on clean surfaces can be used to understand the basic mechanisms of bacterial contamination and biofilm spreading, including the possibility to prevent its formation by inhibiting cell attachment. A different, but not less interesting, approach concerns the interaction of droplets and bubbles on biofilm covered surfaces. The investigation of this type of wetting can be applied in the study of biofilm structure, the prevention of its further growth, and its removal from already contaminated surfaces. A possible application would be the optimization of cleaning solutions and detergent formulations. We should mention a particular case would be that of the interaction of surface pre-contaminated by a biofilm with a droplet contaminated with a different cell line (eventually more dangerous respect to the original host). Biofilm coated surfaces can represent a favourable environment for further contamination, for this reason biofilm removal is always recommended, even in the case of non-dangerous contaminations.

Although the majority of actual biofilms are hydrophilic -due to hydrophilicity of EPS- there is not a single study on their wetting properties. The argument behind it is that hydrophilic biofilms can be easily removed so no concern exists on their wettability. Even though, bacteria motility, biofilm superficial properties and their mechanical properties, influenced by their morphology, are of a great importance in predicting the biofilms behaviour and removal. The comprehension of their wetting

behaviour may serve as a tool to better understand their structure. This can be done not only by using static wetting properties, as in the case of hydrophobic biofilms, but also by testing their dynamic behaviour in the spirit proposed in [100] for a patterned surface. Another important issue regarding wetting of biofilms is that the interaction of biofilms with bubbles has also not been studied. This may have practical interest since it has been proposed that introducing bubbles in cleaning water enhances its biofilm removal properties [101]. A wider investigation of this marginally studied aspect is needed. Figure 5 summarizes the main directions for future research efforts regarding biofilm wetting according to the present authors point of view.

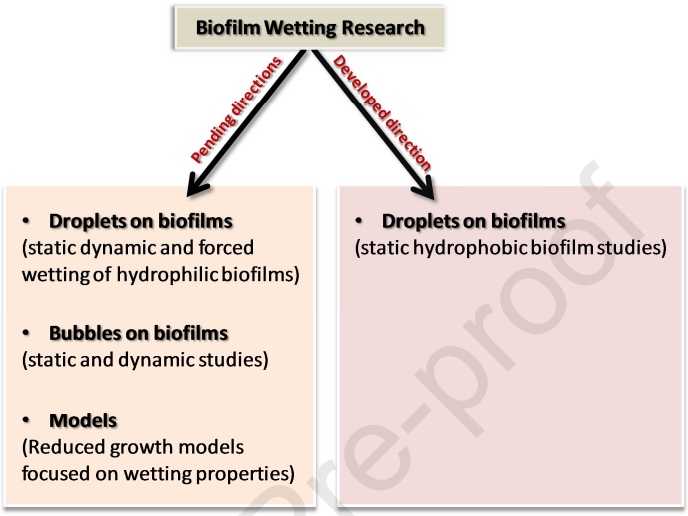


Figure 5 A schematic on the current and future research topics regarding biofilm wetting.

Moreover, knowing that biofilms are formed by reversible and irreversible attachment of cells to a solid substrate, followed by microcolony formation, maturation and detachment, motility of biofilms should be subjected to studies in the flow condition as it can induce formation of aggregates and affect interfacial properties. Bacterial motility can heavily affect interfacial properties also in the case when bacteria are present in a droplet of liquid wetting a surface. Additionally, considering that biofilms are inhomogeneous porous films, the porous medium, soft/deformable substrates could be used as models in investigation of wetting properties of the biofilms.

Finally, having substantially understood the chemical effect on wetting resistance of biofilms the next step is to further examine their structural effect [102]. In this respect, extensive BS biofilm structural characterization is conducted, using SEM images and light profilometry, and an attempt is made to correlate the resulting parameters to the wetting behaviour. Depending on the nutrient type and location on the colony, three different wetting regimes are identified. The two are of non-wetting nature and through correlation to the structural biofilm characterization it is argued that the one is of lotus-leaf (Cassie-Baxter state) type and the other of rose petal (impregnated Cassie state) type. Very interestingly, the realized state is affected by the nutrient availability. The next reasonable research step is to find ways to overcome the wetting resistance of certain biofilms [12]. Extensive experiments and measurement of topological structural parameters and contact angle for biofilm created by 5 types of bacteria are performed in [12]. The correlation between surface roughness, in terms of developed interfacial area ratio index, of biofilm and its contact angle is clearly presented.

The above observation motivated the following hypothesis: if the roughness features of a highly complex biofilm surface could be smoothened, such a biofilm surface should lose its strongly

hydrophobic character. In this respect it is found that a short treatment with ethanol solutions renders omniphobic biofilms omniphilic. It is also shown that the same e□ect can also be obtained by using less aggressive chemicals such as concentrated salt and sugar solutions.

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Declaration of interest: none

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The idea of modifying the biofilm wetting properties by modifying its surface topology is tested. It is found that after treatment with appropriate topology altering agents, the biofilm can be transformed from hydrophobic to hydrophilic enhancing the efficiency of antimicrobial solutions for its removal. Also, The effect of topographical changes of the biofilm surface on the wettability

of these surfaces is demonstrated. These types of alterations were done by surface exposure toward different solutions.
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999 for the first time in the standard context of Interfacial Science regarding wetting modes.

Wetting/Spreading on Porous Media and on Deformable, Soluble Structured Substrates as a Model System for Studying the Effect of Morphology on Biofilms Wetting and for Assessing Anti-Biofilm Methods

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