Research & development

(BIO)FOULING AND ANTIFOULING MEASURES

Initial phase of biofouling: the microbial biofilm formation

The biofouling formation is a sequential process that starts with the adsorption of organic macromolecules (proteins, glycoproteins and polysaccharides). The second step, is characterized by the adhesion of prokaryotes and the subsequent development of a bacterial biofilm starting to produce a matrix of Extracellular Polymeric Substances (EPS). Here we will discuss how the bacterial community composition can be assessed during the initial phases of the biofilm development by the CAtalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD–FISH), in combination with Confocal Laser Scanning Microscopy (CLSM).
Understanding the first steps of the biofilm development process is of crucial importance for micro and macro fouling control and prevention

DOI: 10.12910/EAI2014-40

Stefano Fazi, Ilaria Pizzetti, Francesca Di Pippo

Introduction

In the marine environment, all immersed surfaces (natural or artificial) are rapidly colonized by a succession of organisms, the outcome being known as 'biofouling' (Figure 1). Within the first hours, surfaces are covered by microbial biofilms (microfouling) [1], that influence the successive settlement, colonization and growth of macroorganisms (macrofouling) [2]. Biofilm formation is then followed within a week by diatoms (microalgae), spores of macroalgae (seaweeds), protists, fungi and protozoa, followed in turn by larvae of invertebrates such as barnacles (Linear successional surface colonization model) [3, 4, 5, 6, 7].
The implications of microbial biofilms in marine biology, and especially in relation to biofouling, have also been extensively studied, and a wide type of specific interactions between microbial biofilms (Figures 1 and 2) and fouling organisms [8, 9, 10, 11, 12] have been revealed. Microbial biofilms were shown to influence the settlement of marine organisms decades ago [13]. More recently, the formation, composition and physiology of bacterial biofilms have been studied, including their role in the environment [14, 15, 16, 17]. It is now well established that density-dependent, cell-to-cell communication processes between bacteria, generally referred to as 'quorum sensing', control several important features of biofilms (e.g., development, virulence and dispersal stages) [18, 19, 20, 21].

Stefano Fazi, Ilaria Pizzetti
Water Research Institute - National Research Council (IRSA-CNR), Monterotondo – Rome

Francesca Di Pippo
Water Research Institute - National Research Council (IRSA-CNR) Institute for Coastal Marine Environment - National Research Council (IAMC-CNR), Sa Mardini, Torregrande, Oristano

Initial stage of biofilm development: bacterial colonization, matrix formation and maturation

Bacteria are considered to be the primary colonizers of substrata, constituting the initial stage of biofilm development. By encountering surfaces, free-swimming microbial cells can switch from a planktonic to a benthonic lifestyle exuding a slimy matrix and forming complex and dynamic communities with high phenotypic diversification and high degree of cellular coordination [22].

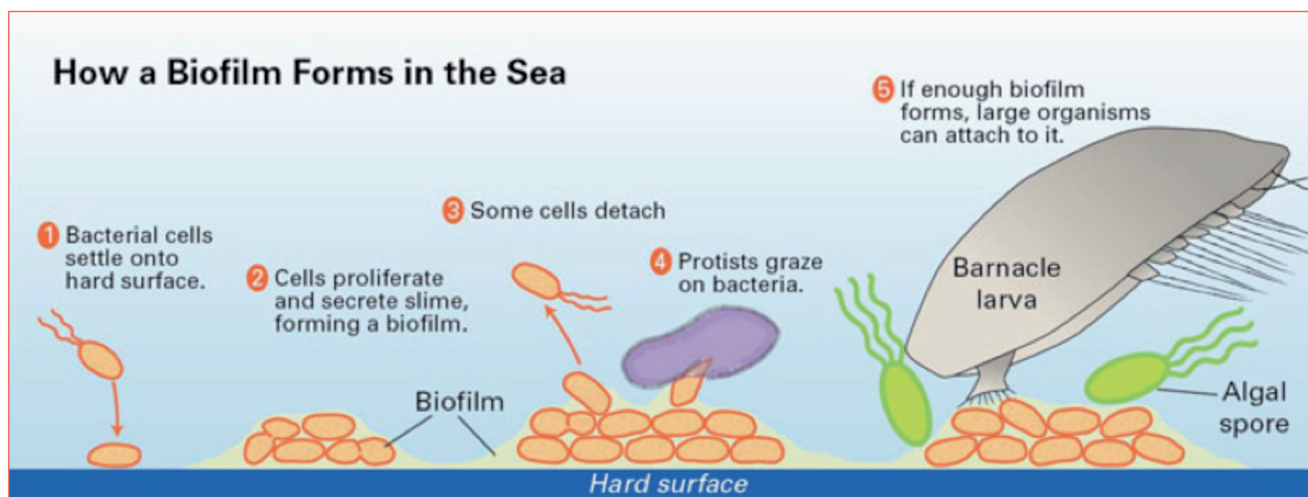

FIGURE 1 Microbial biofilm formation. Modified from http://www.whoi.edu/oceanus/illustrations

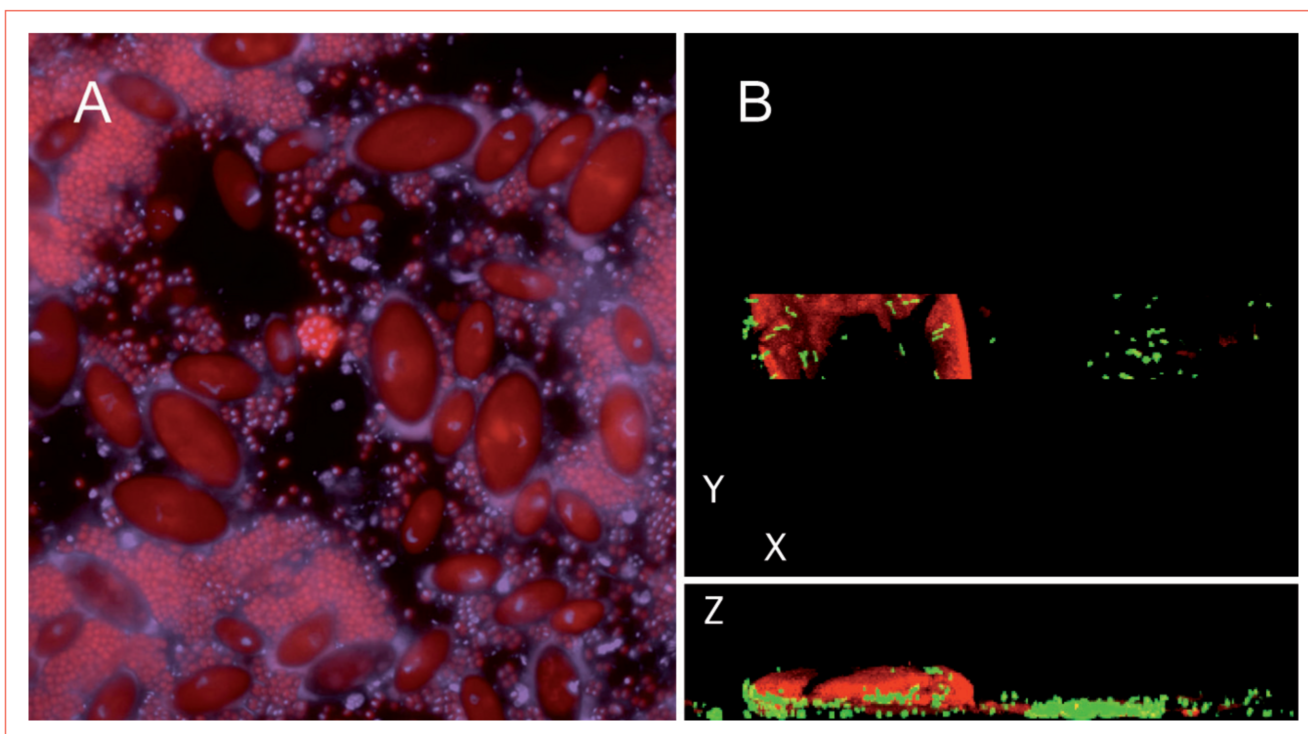

FIGURE 2 A) Epifluorescence micrograph of biofilm. Blue, DAPI signals of bacteria; the red signal was due to the Chlorophyll a autofluorescence in cyanobacterial and microalgal cells. B) CLSM images showing the spatial distribution of bacteria (X-Y plane) and the biofilm thickness (X-Z plane), as determined by CARD-FISH. The autofluorescence of the photosynthetic pigments (Chl a) was detected with the 633-nm line of an Ar/HeNe laser (excitation) and observed in the red and far-red channels at 590 to 800 nm (emission). The hybridized cells were excited with the 488-nm line of an Ar laser and observed in the green channel from 490 to 530 nm (adapted from Lupini et al. [39])

Biofilms are now recognized as matrix-enclosed, attached microbial communities that can develop highly differentiated architectures, including mushroom-like structures, ripples and ridges, or filamentous streamers floating in the bulk liquid. The extracellular matrix is a key factor for the overall biofilm functionality. It is a highly hydrated system composed of extracellular polymeric substances (EPS) comprising exopolysaccharides, along with a wide variety of proteins, nucleic acids, glycoproteins, phospholipids, glycolipids and humic substances [14, 15, 23, 24]. Although the precise and molecular interactions of the various secreted biofilm matrix polymers have not been defined, and the contributions of these components to matrix integrity are poorly understood at the molecular level [25], several functions of EPS have been determined. Independent of the EPS composition, the matrix typically features a hydrogel-like structure, which embeds the biofilm cells and determines the physico-chemical and biological properties of the whole biofilm [26, 27].

The matrix network encloses and holds together the microorganisms in the biofilm, providing mechanical stability to the community [26, 27], which is the major advantage of the biofilm mode of life for microorganisms [28]. In addition, EPS are thought to play an important role in the adhesion of cells to substrata. This allows the formation of stable and functional microconsortia with a low expense of energy, allowing cells to metabolise, reproduce and communicate between each other more efficiently [29]. In addition to the advantages of mechanical stability, the matrix also provides protection against heavy metals, other toxic substances and grazing by predators. The intense research on single- or multi-species biofilms grown in flow cells have also unravelled many microbial interactions (competition, cooperation), largely deterministic in nature, due to the coexistence of niche differentiation [22].

In the wild, biofilms are open and dynamic communities and are part of a larger network; some authors suggested a new ecological concept of biofilms, and by viewing biofilms as microbial landscapes, studied their community assembly according to the metacommunity ecology theory [19, 22, 30]. The formation of phototrophic biofilms is a complex process, regulated by diverse hydrodynamic and chemical characteristics of the surrounding water, preconditioning of the substratum, cell surface characteristics, EPS secretion [30, 31, 32]. As biofilms develop, competition for resources such as nutrients, light, and space, is believed to select those species that are more competitive for a limiting resource. Oxygenic phototrophic microorganisms such as benthic diatoms, unicellular and filamentous cyanobacteria, and benthic green algae generate energy and reduce carbon dioxide, providing organic substrates and oxygen. This photosynthetic activity fuels metabolic processes and conversions in the entire biofilm community, including the heterotrophic fraction [33].

The utilization of CO_2 during photosynthesis results in steep vertical redox and chemical gradients that enforce the stratification in these communities along the microenvironments, restricting phototrophic microorganisms to the upper layer of the biofilm, most anoxygenic phototrophs and anaerobic chemotrophs to the lower part. With the increasing complexity of maturing biofilms, competition for resources is likely to support high species diversity and spatial heterogeneity, as a result of concurrent functional niche diversification within the biofilm [19].

Single-cell approach and CLSM to study the biofilm 3D architecture

Currently, increasing attention is being paid to biofilms that develop on artificial substrata immersed in seawater [34, 35, 36, 37, 38]. However, microbial biofilms in aquatic environments are very heterogeneous and dynamic systems, which makes them difficult to model and investigate. In marine biofilms developed on unpainted artificial surfaces, microbial communities mainly consist of bacteria and diatoms [39]. Proteobacteria, especially a-proteobacteria, appear dominant among these bacterial communities [40, 41, 42, 43], but the population dynamics depends on several environmental factors. Marine biofilm communities have also been reported as a potential source of pathogenic bacteria [44, 45]. However, bacterial communities grown on dissimilar surfaces appeared to evolve and become more similar over time, as determined by Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescence In Situ Hybridization (FISH) [41,42]. By

using Fluorescence In Situ Hybridization techniques (e.g. CARD-FISH), the bacterial community composition can be documented, but loosing information on the spatial distribution of specific bacterial clusters, which is due to the destruction of the biofilm structure by scraping and filtering [46, 47, 48]. When it comes to the possibility of visualizing specific cells while maintaining the 3D structure of the biofilm unaltered, there have been substantial improvements made by utilizing FISH in combination with Confocal Laser Scanning Microscopy (see CLSM-FISH in [49]). A limited number of studies have demonstrated the direct use of CLSM-FISH on a biofilm attached to an artificial or natural substratum (e.g. polycarbonate slides – [50]; clay beads – [51]; polystyrene beads – [52]; marine algae – [53]). Several attempts have recently been made by embedding biofilms on gel pads [54] or by using crio-sectioning [55, 56, 57, 58]. However, such additional manipulation can potentially lead to a loss of mass and/or distortion of the in situ perspective [52].

We optimized a straightforward CARD-FISH protocol in combination with CSLM for the hybridization and the inspection of biofilms attached to the original substrate [59]. Thus, the protocol allows the simultaneous identification and the spatial localization of cells, while maintaining the natural architecture of the biofilm unal-

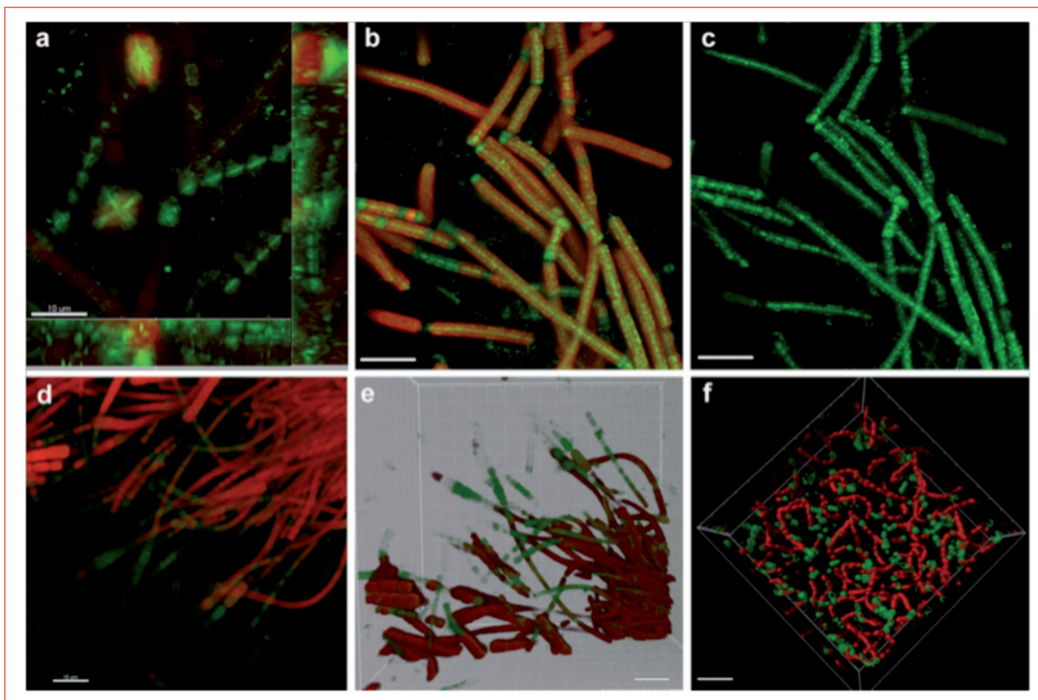

FIGURE 3 CLSM images after staining with fluorochromes. (a) Reaction of *Anabaena augstumalis* biofilm to concanavalin A-Alexa Fluor 488-conjugate, showing neutral polysaccaridic material (green signal, Capsular Polysaccharides matrix-forming) deposited around the vegetative cells and akinetes, where filaments attached to the substratum; (b, c) fine neutral polysaccharidic envelope surrounding the filament of *P. autumnale* biofilm; (d, e) *Calothrix* sp. biofilm after staining with concanavalin A-Alexa Fluor 488-conjugate, showing the positive reaction of the basal part of the filament and the 3D reconstruction image of the filaments of *Calothrix* sp. biofilm and the envelopes around the basal part; (f) 3D reconstruction of the reaction of *Nostoc* sp. biofilm with concanavalin A-Alexa Fluor 488-conjugate, showing the presence of an envelope around the heterocysts (green). The red signal was due to the Chl *a* autofluorescence in vegetative cells and akinetes. Scale bars: 10 μm in a-c and 15 μm in d-f (adapted from Di Pippo et al. [19])

tered. Due to the impracticality of applying the CARD-FISH protocol to the biofilm grown on flat microscope glass slides, traditionally used for the analysis of the epiphytic community in flowing water systems [60], we utilized chambered slides (10-well diagnostic microscope epoxy coated slides; well diameter: 6.7 mm - Thermo Scientific, Germany). The slides were randomly collected in triplicate and then fixed in formaline (2% final concentration). The slide surfaces around the wells were gently cleaned with a small tampon to remove the biofilm grown in-between the wells, thus avoiding buffer scramble during the procedure. CARD-FISH was performed, optimising the protocol for the analysis of bacterial cells on polycarbonate membrane after sample filtration described by Fazi et al. [61, 62] (for details, see [59]).

CLSM can also be utilized to study the following stages of microfouling, when microalgal and cyanobacterial microconsortia colonize the bacterial layers [63], allowing the formation of phototrophic biofilms. Confocal microscopy provides information on the morphology of the biofilm-forming microorganisms, their spatial distribution, relationships with substrata and the interactions among microbial members. The use of CLSM in a multichannel mode allows the visualization of the spatial distribution of cyanobacteria and associated microalgae, bacteria and archea in phototrohic biofilms as well as the distribution of EPS components by collecting series of optical sections at the appropriate excitation and emission wavelengths (Figure 3). The different channels map individual biofilm components, detecting differences in the biofilm-forming phototrophic cells thanks to their specific autofluorescence, due to their intrinsic content in chlorophylls and phycobiliproteins absorbing in different wavelenghts. The superimposition of optical sections results in 2D and 3D images that show the cellular and sub-cellular heterogeneous distribution along the biofilm. Since the CLSM techniques guarantee the structural integrity of biofilm communities, it is possible to evaluate the distribution of the different exopolymers that constitute the matrix by using different fluorochromes to bind glycoconjugates, proteins and nucleic acids. We used different fluorochromes on monospecific cyanobacterial biofilms at the initial stage of development, and the CLSM observations have shown neutral exopolysaccharides specifically deposited within the envelope around the cells, especially where filaments attach to the substratum. Our results, based on CLSM observation, highlights how the diverse compositions of exopolysaccharides surrounding vegetative cells reflect the different roles of polymers at different positions (Figure 3).

Conclusion

Bacterial successional changes can be described by applying the CARD-FISH protocol to intact biofilms, thereby avoiding biofilm detachment or manipulations. Our approach, in combination with an appropriate spatial analysis, could contribute to elucidate how specific bacterial clusters participate in the development of the complex biofilm structures and the mechanisms that regulate community composition dynamic and cell dispersion in aquatic environments. Moreover, thanks to the intrinsic content in pigments of phototrophic cells and the use of fluorochromes, EPS-binding is possible to obtain information on spatial distribution of cyanobacterial, algal and exopolymeric components of phototrophic biofilms. These technologies help to understand the first steps of the biofilm development process for micro-and macro-fouling control and prevention.

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