STUDIES REGARDING THE FORMATION AND TEMPORAL DYNAMICS OF BACTERIAL BIOFILMS ON THE HYDROPHILE SURFACE OF GLASS IN STATIC AND DYNAMIC CONDITIONS

AURELIA MANUELA MOLDOVEANU[1], IOAN I. ARDELEAN [1, 2]

[1]*Ovidius University of Constanta, 900527, Constanta, Romania*
[2]*Biology Institute of the Romanian Academy, 060031, Bucharest, Romania*

Abstract. *The biofilms represent communities of prokaryotes and eukaryotes that are formed in different types of hydrophile and hydrophobe surfaces determining the occurrence of microfouling, biocorrosion and the reduction of materials efficiency. The generation of biofilms was accomplished based on a comparative study using seawater as culture medium and glass slides as artificial support for the adherent cells. Thus, three experimental systems were used (containers, bioreactor and aquarium), which permitted the use of static and dynamic study methods. The temporal dynamics of a biofilm is accomplished quickly. Only three days after the immersion of the artificial substrate, a dense layer of cells is formed on the hydrophile surface of glass and 15-21 days later, depending on the thickness of the biofilm, detachment and dispersion phenomena occur. The formation of biofilms in static conditions occurs more quickly from density of $12 \cdot 10^2$ cel/mm^2 to $59 \cdot 10^2$ cel/mm^2, because the speed of water recirculation influences the number of adherent bacteria.*

Keywords: *biofilm, bioreactor, fouling, biocorrosion, static and dynamic methods*

1. INTRODUCTION

In the conditions of marine environment, the immersion of materials with hydrophile or hydrophobe molecules may determine the formation of multilayered communities of prokaryote and eukaryote cells known as biofilms [1]. The biofilms have major negative effects in different science domains, with multiple implications in microbiology, medicine, industry, materials engineering etc. Their study is approached from different perspectives, especially regarding the control of their occurrence because they clog filters, and reduce the efficiency of biomaterials (catheters, rods etc) and materials by the formation of fouling, which is difficult to eliminate from the harbor zones and which determines biocorrosion and biodegradation [2].

The formation of biofilms at the level of the liquid-solid interference is mediated by the existence of anfractuosities in the substrate and the presence of nutrients at the level of the interference, which determines the positive chemotaxis of prokaryotes towards the substrate, but also by the existence of the extracellular matrix by which cells attach themselves irreversibly to substrates [3]. The formation of a biofilm occurs under the direct influence of environmental factors, among which water dynamics has a very important role, especially “in situ”, where the currents can influence the number of cells attached to surfaces [4].

In “in vitro” conditions, building systems for the study of biofilms with a mechanism for the recirculation of seawater (bioreactor) can allow the obtaining of data in similar conditions to those in the natural environment, compared to the static methods in containers, used frequently for the study of biofilms [5].

Considering the great practical importance of the formation of bacterial biofilms in seawater, the purpose of this paper is to determine the density of bacterial cells during the formation of the biofilms, the temporal dynamics and the possible influence of water kept in aquarium conditions in the quicker formation of bacterial biofilms in the laboratory.

2. MATERIALS AND METHODS

In our experiments, we investigated the formation of biofilters in seawater in three experimental versions: static conditions (in containers and aquarium without water recirculation system) and dynamic conditions using bioreactors. All the experiments were accomplished at a constant temperature of 18ºC in the thermally regulated room of the Laboratory for Biodiversity Investigation within "Ovidius" University of Constanta. The hydrophile surfaces used for the analysis of the biofilms were represented by 296 glass microscope slides, previously degreased with 70% ethanol and sterilized by heating at 180ºC in the drying oven for one hour in order to avoid contamination with microorganisms and organic matter prior to the experiment [6].

In order to obtain biofilms on the smooth glass surface, we used as culture medium seawater from the Black Sea littoral and seawater kept in "in vitro" conditions in an aquarium [7] without water recirculation system in the Laboratory for Biodiversity Investigation. The mentioned media can be used to obtain biofilms because of the planktonic bacteria dispersed in the liquid stage.

First we tried to obtain bacterial microfilms in static conditions "in vitro" using sterile plastic containers (100 ml) in which the slides were introduced according to the adapted Henrici method, where the slides were positioned in an oblique position, compared to the classical method with horizontal slides, in order to avoid the sedimentation phenomenon [8].

A second experimental system was represented by the four bioreactors used to recreate the dynamic conditions "in vitro" which were accomplished according to the model suggested by Hansen et al. [9]. Thus, we used PVC boxes (15 l) to which aquarium pumps (200l/h) were attached to ensure the continuous water circuit on the experimental surface.

In the bioreactors, 2.5 l of seawater were used as culture medium, above which 12 glass slides were arranged in an oblique position and attached to 2 plastic rods located on a rack. Thus, the glass slides came into contact only with the flowing seawater, according to the bioreactor method and the *microbial fishing* method [9].

The aquarium used in the experiments (capacity = 20 L) contained seawater from the harbor zone and mussel colonies, which are organisms with role in the formation of microfouling, whose generation is favored by the presence of bacterial biofilms on artificial surfaces. The slides completely immersed in the aquarium water were placed obliquely and attached to the two plastic rods according to the *microbial fishing* method [8].

In order to obtain data about the temporal dynamics of the biofilms, these were investigated for 21 days, with the harvesting of the slides every three days. The initial phases of biofilm formation were watched for two days, with the harvesting of the slides at certain hour intervals. On the first day, the slides were harvested hourly for 12 hours for the containers (10 hours for the bioreactors). Subsequently, the slides were left in containers/bioreactors over night for 12 hours and the collection was resumed the following day at an interval of two hours for a period of 12 hours for the containers (10 hours for the bioreactors).

After harvesting, the slides were subjected to a chemical fixing process for 30 minutes in 2.5% formaldehyde solution in artificial seawater obtained by mixing 18g of marine salts with 1 liter of osmosis water in order to obtain the average salinity of the Black Sea (18 g/L). Afterwards, they were subjected to a process of desalinization by washing for 10 minutes in three successive solutions made up of: the first - 75% artificial seawater with 25% osmosis water, the second - 50% artificial seawater and 50% osmosis water, and the last one - 100% osmosis water [10].

After desalinization, the slides were immersed for one minute in 1.5% gentian violet solution in 10ml ethanol 70%. Subsequently, the volume was brought to 100ml by adding

distilled water according to Sonak [11], washed twice with osmosis water and left to dry at room temperature [10].

The slides were analyzed under Hund Wetald microscope in bright field with 50X objective and 10X ocular. The number of bacteria was determined by means of the 10 mm X 10 mm micro-ocular grid (macroscopically), investigating 20 microscopic fields per harvested slide. The calibration of the micro-ocular grid was realized for the objective and ocular mentioned previously and it was determined that the grid image on the microscopic field is $0.04mm^2$, a surface which represented the area for counting the cells attached to the glass slide for each microscopic field analyzed.

3. RESULTS AND DISCUSSIONS

In order to emphasize whether there are differences between the two types of culture media used for the generation of the bacterial biofilms on the hydrophile surface of the glass slides, chemical analyses of the seawater were accomplished in the Chemistry Laboratory within the "George Antipa" Institute for Marine Research of Constanta (Table 1).

Table 1. The values of the chemical parameters of seawater (liquid culture medium)

Chemical parameters	Sea water (littoral)	Sea water (Aquarium)
salinity	15.11 g/L	25.17 g/L
pH	8.17 unit.	6.86 unit.
$P\text{-}PO_4$	0.77 µmoles/dm^3	65.80 µmoles/dm^3
$N\text{-}NO_2$	0.40 µmoles/dm^3	13.41 µmoles/dm^3
$N\text{-}NH_4$	1.09 µmoles/dm^3	4.46 µmoles/dm^3
$N\text{-}NO_3$	3.17 µmoles/dm^3	29.25 µmoles/dm^3
$Si\text{-}SiO_4$	22.16 µmoles/dm^3	0.19 µmoles/dm^3

The chemical analysis of seawater emphasized the existence of differences in the chemical parameters (salinity, pH, concentration of the inorganic substances) between the two types of seawater used. The seawater in the littoral area has parameters with normal values also recorded in the previous years [12]. But the values of the seawater from the aquarium are considerably over the normal limit, displaying an increase of salinity of over 10g/L and a decrease of pH by two units, compared to the littoral seawater.

The concentration of inorganic substances is much higher than the normal one for seawater, the amount of nitrates being three times higher than the normal one, while the amount of phosphates was over 84 times higher. The existence of these differences between the two culture media used may determine changes in the formation manner and the temporal dynamics of the generation of bacterial biofilms in liquid medium.

The analysis in bright field of the biofilms formed on the hydrophile surface of the glass slides collected from the containers with littoral seawater and of the slides immersed in an aquarium emphasized the existence of successive phases for the formation of biofilms, which display an important increase of the bacterial density (Fig. 1) after a period of only three days from the immersion of the substrate into seawater.

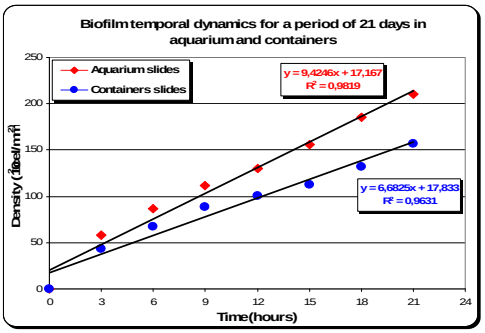

Fig.1 The temporal dynamics of the biofilms for a period of 21 days.

Thus, the density reaches a value of $43 \cdot 10^2$ cells/mm^2 after only three days in the case of the seawater containers. This value doubles to $89 \cdot 10^2$ cells/mm^2 9 days later and increases progressively up to 21 days when the cellular density triples to $157 \cdot 10^2$ cells/mm^2.

For the slides immersed in the aquarium, there is an increase of cell density from $58 \cdot 10^2$ cells/mm^2 to a double of $112 \cdot 10^2$ cells/mm^2 10 days later and a progressive increase up to 21 days when there is a tendency for the tripling of the value of adherent cells density.

There are differences between the results obtained in the two experimental versions, the values obtained for the biofilms formed in the aquarium conditions by direct immersion being higher due to the lack of the water recirculation system which facilitates the sedimentation phenomenon and microorganism attachment, but also the attachment of small mussels which become fixed to the slides by means of byssus. This demonstrates that the bacteria mediate the colonization of the artificial surfaces in the marine environment.

We noticed on slides that during the first stages, small bacteria adhere, especially cocci and bacilli and the micro-colonies occur. Afterwards, spirilla bacteria adhere and also bigger forms such as pedunculate bacteria. This offers the aspect of complex structure made up of prokaryotes and eukaryotes such as diatoms and chlorophycea. These observations are confirmed by Compère's data [2] who observes the existence of a multilayered biofilm 21 days after the surface immersion. This biofilm is made up of various bacteria attached to the matrix of exopolysaccharides and with bacterial densities of 10^7cells/cm^2, values higher than those obtained for the bacteria at the Romanian littoral.

The seawater kept in aquarium conditions determines an increase of the number of cells present, but also of the eukaryote algae, especially of the diatoms present in the planktonic stage. This is due to the lack of a seawater recirculation system, but also to the presence of pumps, the attachment of bacteria occurring more easily, a fact mentioned by Hovanec [7].

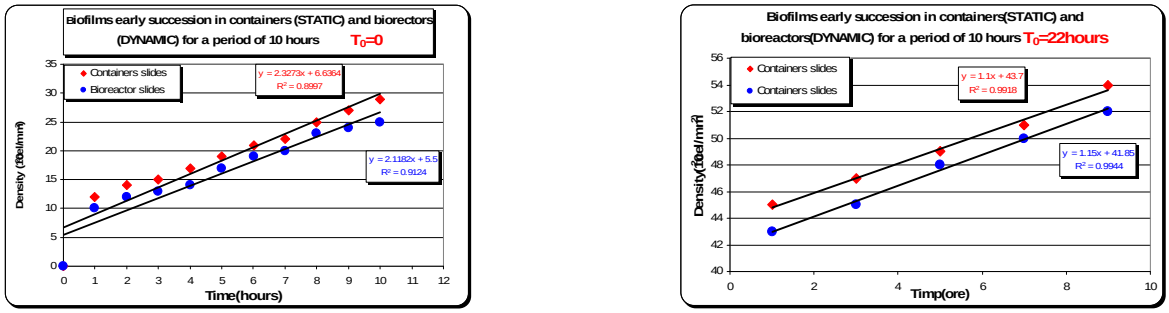

Fig. 2 The initial stages in the formation of a biofilm in static and dynamic conditions using littoral seawater as culture medium.

The glass slides collected from the containers and bioreactors were analyzed under microscope and after counting the fields, a progressive increase of the cellular density was

observed in the case of the biofilms formed in the seawater containers, from $12 \cdot 10^2$ cells/mm^2 one hour after immersion to the doubling of the cellular density eight hours later to $25 \cdot 10^2$ cells/mm^2 and the tripling of its value 16 hours after immersion (Fig. 2).

The rapid rhythm of biofilm formation in static conditions may be observed through the rapid formation of microcolonies in the first hour from the immersion of the slides into liquid medium. The microcolonies are made up especially of cocci and bacilli, results confirmed by Meritt [13] who mentions that the formation of microcolonies is more favored by the static conditions during the first hours than by the dynamic conditions. In the case of the littoral seawater recirculated in bioreactor conditions, a slower increase was noticed compared to that in static conditions, with density values of $10 \cdot 10^2$ cells/mm^2 one hour after immersion and a double value of $19 \cdot 10^2$ cells/mm^2 six hours after immersion, but also a triple value to $29 \cdot 10^2$ cells/mm^2 12 hours after immersion (Fig.2).

The microcolonies are also formed in dynamic conditions, but in a slower rhythm which depends on the speed of the water flux, in this case a high flux of 200l/h which determined the formation of colonies three-four hours from the immersion of the substrate into liquid medium. The colonies are made up especially of bacilli fixed in the matrix of exopolysaccharides.

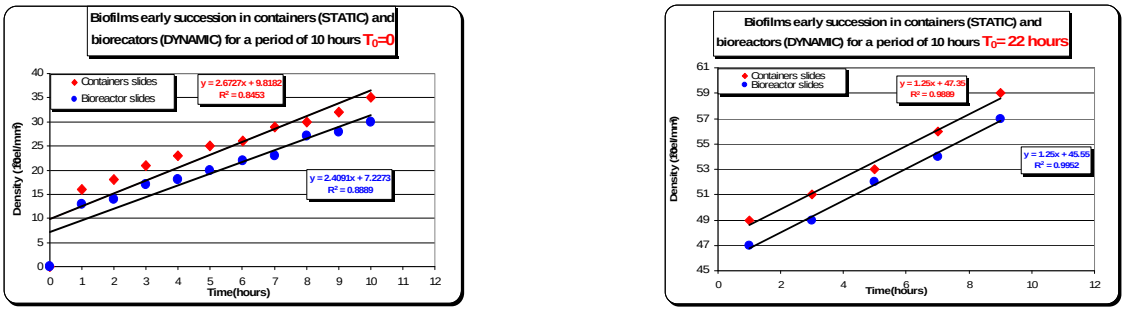

Fig. 3 The initial stages in the formation of a biofilm in static and dynamic conditions using aquarium seawater as culture medium.

In the case of the slides collected from the containers and bioreactors in which aquarium seawater was introduced, there was a progressive increase of the cellular density in the case of the biofilms formed in static conditions in containers, from $16 \cdot 10^2$ cells/mm^2 one hour after immersion to a double value nine hours later ($32 \cdot 10^2$ cells/mm^2) and a triple value ($59 \cdot 10^2$ cells/mm^2) 36 hours after immersion. These values are higher than those recorded in the case of littoral seawater in the same conditions (Fig. 3).

For the bioreactors in which aquarium water was used, the density value increases from $13 \cdot 10^2$ cells/mm^2 one hour after immersion to a double value ($27 \cdot 10^2$ cells/mm^2) nine hours later and a triple value ($56 \cdot 10^2$ cells/mm^2) 32 hours after immersion, the values recorded are higher than those in the bioreactors with littoral seawater, in the same conditions, due to the differences in the chemical parameters of the seawater used in experiments (Fig. 3).

The values of cellular density obtained in our experiments were lower compared to those obtained by Risnaarts at al. [3] in similar conditions. They observed an increase of the bacterial density between $5 \cdot 10^4$ cells/cm^2 and $1.6 \cdot 10^7$ cells/cm^2 in static conditions and between $5 \cdot 10^4$ cells/cm^2 and $3.6 \cdot 10^7$ cells/cm^2 in the dynamic conditions. The considerable difference can be given by the water recirculation speed. When this process is slower, the biofilm cells receive more substances but they are also offered better attachment conditions by the adherence mechanisms on the experimental surface.

This kind of experiments in static and dynamic conditions was accomplished “in situ” by Casse and Swan in [4]. They observed a larger percentage of bacterial density (80%) in static conditions compared to those formed in dynamic condition on different types of

immersed surfaces in the conditions of marine environment without the application of anti-fouling substances.

4. CONCLUSIONS

The numeric differences existing between different types of experimental versions and the culture media used demonstrate that seawater in static conditions favors the formation of bacterial biofilms, compared to the dynamic conditions, especially if the speed of the water flux is high and prevents the attachment of marine bacteria through the adherence mechanisms. The use of seawater kept in aquarium conditions as liquid culture medium may facilitate the more rapid formation of biofilms and fouling in the presence of an excess of nutrients and in the absence of a recirculation system.

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