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PRELIMINARY STUDIES REGARDING CELL DENSITY AND TEMPORAL DYNAMICS OF BACTERIAL BIOFILMS FORMED AT LIQUID - SEDIMENT INTEFACE USING BRIGHT FIELD AND EPIFLUORESCENCE MICROSCOPY

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Key words: *biofilms, slides, static condition, hydrophile, buried slide, microbial capture*

INTRODUCTION

The evaluation of microbial processes at surfaces has, to a large extent, been dependent upon microscopic examination, indirect analysis of bulk aqueous-phase properties, or analysis of the biofilm following its removal from the substratum. While these techniques provide useful information on colonization dynamics, biofilm structure, and the impact of biofilm processes on the adjacent bulk aqueous phase, they offer little insight into the chemistry of the biofilm matrix or the underlying substratum surface (Bendinger et al., 1993; Costerton, 2007).

The surface immersed in the marine environment becomes almost instantaneously a favorable medium for the attachment of a layer of polymers from the environment, a phenomenon that determines the adhesion of microorganisms and the formation of biofilms with negative impact on the surfaces in industry, medicine, alimentation and environment. (Merritt et al., 2005; Casse et al., 2006). A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix.

The existence of bacteria in the biofilm stage has certain advantages among which not only the concentration of nutrients on surfaces, but also a postponing of the diffusion of exopolymers in relation to the cell, which thus favors the extra-cellular hydrolysis of the substances.(Zarnea, 1994; Costerton et. al. ,1994; Lazar 2003). The biofilms formed on surfaces are organic and their formation begins in the first few minutes from the exposure of the substrate to seawater, but their evolution is accomplished in a prolonged growing process (Donlan, 2002). It seems the attachment represents an important strategy of bacterial resistance to starvation in extreme conditions, observable at a decrease of the carbon sources even in conditions of laboratory culture.

The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface. (Roszak et al., 1987; Risnaarts et al., 1993).

The solid surface may have several characteristics that are important in the attachment process. Characklis et al. (1990) noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals.

Even though results of these studies have at times been contradictory because no standardized methods exist for determining surface hydrophobicity, some kind of hydrophobic interaction apparently occurs between the cell surface and the substratum that would enable the cell to overcome the repulsive forces active within a certain distance from the substratum surface and irreversibly attach (Fletcher et al.,1979, Bendinger et al., 1993; Pringle et al., 1983).

Considering the great practical importance of the formation of bacterial biofilms in seawater and sediment, the purpose of this paper is to determine the density of bacterial cells during the temporal dynamics of the biofilms formed in laboratory conditions by different microscopy techniques.

MATERIAL AND METHODS

In the experiments the temporal dynamics of biofilms in seawater was investigated in static conditions (in containers). All the experiments were accomplished at room temperature (22º C) in the Microbiology Laboratory within "Ovidius" University of Constanta. The hydrophilic surfaces used for the analysis of the biofilms were represented by glass microscope slides, previously degreased with 70% ethanol and sterilized by heating at 180º C in the drying oven for one hour in order to avoid contamination with microorganisms and organic matter prior to the experiment (Lazar, 2004). To obtain bacterial microfilms in static conditions "in vitro" two sets of sterile plastic containers (100 ml) with 50g of marine sediment and 50 ml of sea water (culture medium) were used.

The slides were introduced according to the adapted Henrici method (fig.1), where the slides were positioned in an oblique position, compared to the classical method with horizontal slides, in order to avoid the sedimentation phenomenon and the buried slide technique (Costerton et al., 1978; www.BiofilmsOnline.com 2008). The slides were positioned with one half in the sea water area of the containers and the other half in the sediment area.

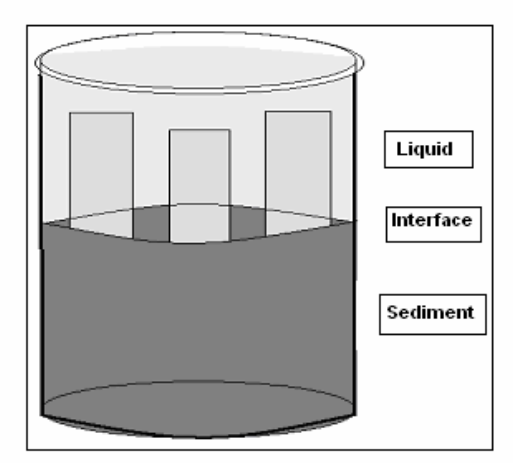


Fig.1. Herici microbial capture /Buried slide technique

To obtain data about the temporal dynamics of the biofilms, these were investigated for 18 days, with the harvesting of the slides every three days.

After harvesting, the slides were subjected to a thermal fixing in the flame for two minutes a process necessary to fix the biofilms on the hydrophilic surfaces of the microscopes slides.

For investigation under the bright field microscopy the slides were immersed for one minute in 1.0% Gentian Violet (Crystal Violet) solution in 100 ml distilled water, and washed twice with osmosis water, and left to dry at room temperature (Rubio, 2002).

The slides were investigated under epifluorescence microscopy using the florochrome Acridine Orange 0,01% and washed twice with osmosis water, and left to dry at room temperature (Rubio, 2002).

The slides were analyzed under Hund Wetzlar microscope in bright field and epifluorescence 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) (Hulea, 1969), investigating 10 microscopic fields per harvested slide.

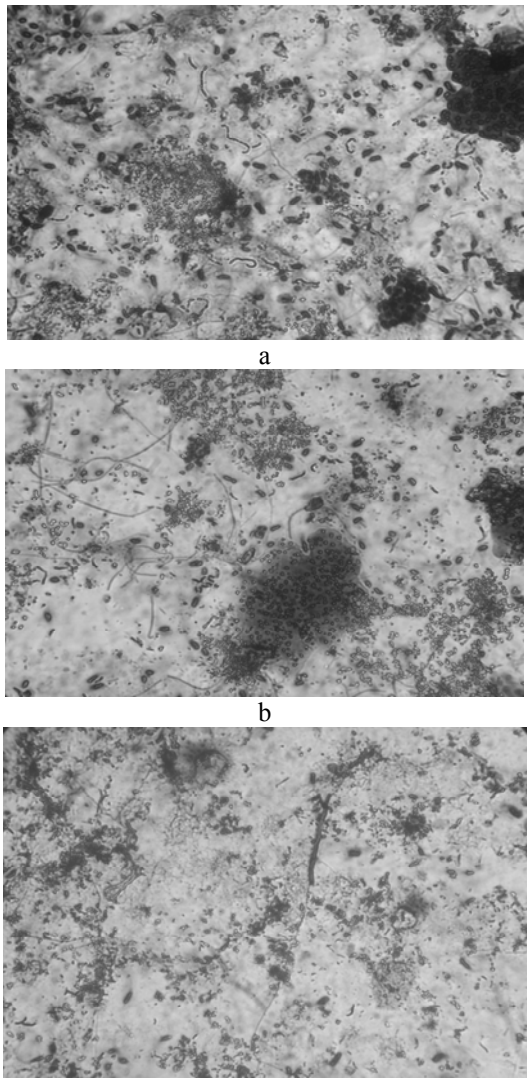
The calibration of the micro-ocular grid was realized for the objective and ocular mentioned previously and it was determined that the grid image on the microscopic field is $0.04mm^2$, a surface which represented the area for counting the cells attached to the glass slide for each microscopic field analyzed (Fry, 1990).

RESULTS AND DISCUSSIONS

Bright field biofilms analysis

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

Crystal violet is also known under the name of gentian violet is a protein dye which stains the bacteria cells in a deep purple color under the bright light microscopy but all sow stains the organic and inorganic matter attach on the slides.



a

b

c

Fig 2. Biofilm formed after 18 days of immersion at interface (a), in sea water area (b) and sediment area (c) (500µm- Bright field microscopy)

Figure two shows the images of biofilms formed in three different slide areas with adhered bacteria, microcolonii, exopolizaharide (EPS) and organic and inorganic matter. In the sea water area there was a high percentage of cell due to the nutrient concentration on the solid surfaces and the high number on bacteria in suspension (fig. 2b), at the interface between seawater and sediment the cell form a thick layer (fig. 2a), visible macroscopically like line and on the sediment area a thin layer was formed and the number of adhered bacteria is much lower than in the other slide areas (fig. 2.c).

It was noticed on the slides that during the first stages, small bacteria adhere, especially cocci and bacilli and the micro-colonies occur (fig. 2 b). Afterwards, spirilia bacteria adhere and also bigger forms such as pedunculate bacteria (fig. 2 a).

This offers the aspect of complex structure made up of prokaryotes and eukaryotes such as diatoms and Chlorophyceae algae.

These observations are confirmed by Compère's data (1999) who observes the existence of a multilayered biofilm 18-21 days after the surface immersion.

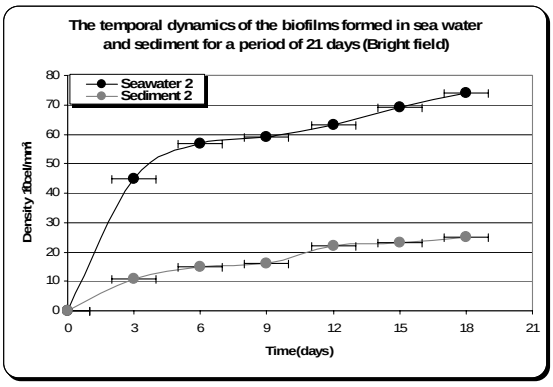


Fig. 3. The temporal dynamics of the biofilms for a period of 18 days

Thus, the density reaches a value of $45 \cdot 10^2$ cells/mm^2 after only three days in the case of the seawater areas of the containers. This reaches a value of $59 \cdot 10^2$ cells/mm^2 nine days later and increases progressively up to 18 days when the cellular density value is $74 \cdot 10^2$ cells/mm^2 (fig. 3).

For the sediment slides areas, there is an increase of cell density from $11 \cdot 10^2$ cells/mm^2 in the first three days to a value of $16 \cdot 10^2$ cells/m ^{2} 9 days later and a progressive increase up to 18 days when the value of adherent cells density reaches $25 \cdot 10^2$ cells/mm^2.The glass slides collected from the containers were analyzed under epifluorescence microscope and after counting the fields.

After the first three days the microorganisms pass the logarithmic phase of growth, and cell density rate is much lower, but the cells divide until the inhibition is reached and the slides are colonized. After this period 15-21 days fragments of biofilms detach from the slides in the environment.

Compere in 1999 in his experiments observe that biofilm is made up of various bacteria attached to the matrix of exopolysaccharides and with bacterial densities of 10^7cel/cm^2, values higher than those obtained for the bacteria at the Romanian littoral because of the different environmental factors.

Epifluorescence biofilms analyzes

The analysis in epifluorescence of the biofilms formed on the hydrophilic surface of the glass slides collected from the containers with littoral seawater emphasized the existence of an important increase of the bacterial density after a period of only three days from the immersion of the substrate into seawater.

Acridine orange is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green).

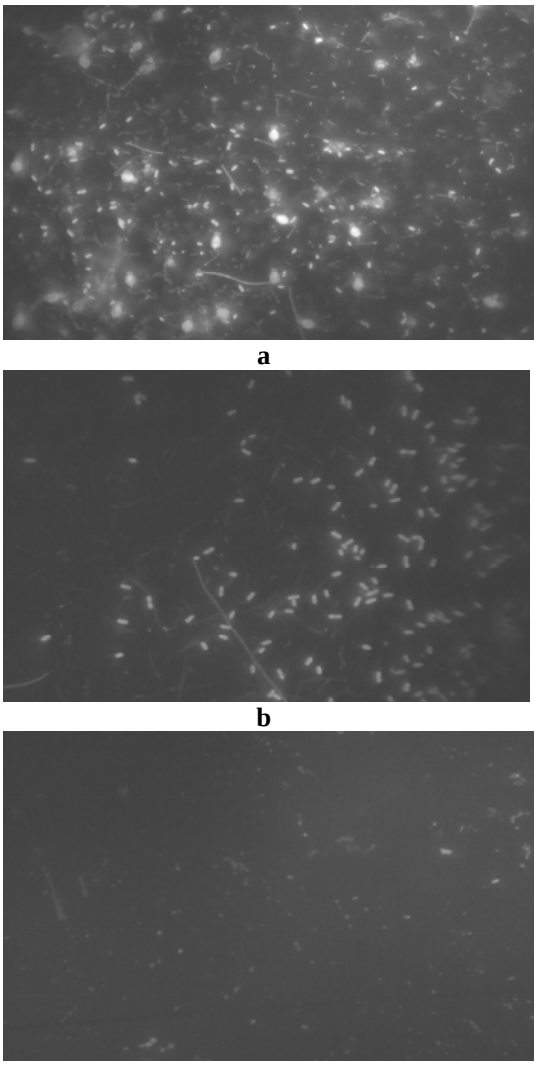
When it associates with RNA, the excitation maximum to 650 nm (red-orange).In figure four are the images of a biofilm formed in three different slide areas with adhered bacteria, microcolonies with exopolizaharide (EPS) the cells are dye in orange or green by the use florochrome and organic and inorganic is not sow individualized.

In the sea water area there was a high percentage of green cell due to the nutrient concentration on the solid surfaces and the high number on bacteria in suspension (fig. 4b), at the interface between seawater and sediment the orange cell form a thick layer (fig. 4a), visible macroscopically like line and on the sediment area a thin layer was formed and the number of adhered bacteria is much lower than in the other slide areas (fig. 4c). Data confirmed by Morató et al. in 2004 how analyzed biofilms by epifluorescence microcopy.

The glass slides collected from the containers were analyzed under epifluorescence and after counting the fields, a progressive increase of the cellular density was observed in the case of the biofilms formed in the seawater areas of the containers, from $45 \cdot 10^2$ cells/mm^2 three days after immersion to value of $56 \cdot 10^2$ cells/mm ^{2}the cellular density after 9 days and later to a value of $74 \cdot 10^2$ cells/mm^2 18 days after immersion (fig. 5).

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 et al. (2005) who mentions that biofim and microcolonies formation is more favored by the static condition.



a

b

c

Fig. 4. Biofilm formed after 18 days of immersion at interface (a), in sea water area (b) and sediment area (c) (500µm-Epifluorescence microscopy)

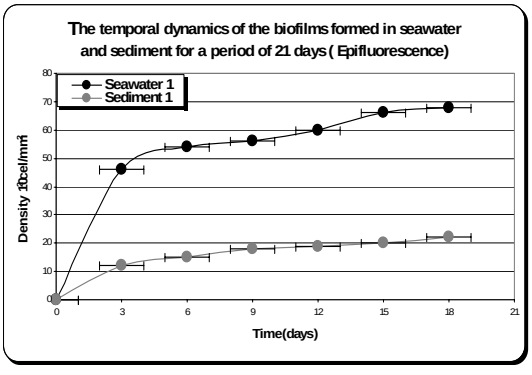


Fig. 5. The differences between the temporal dynamics of the biofilms for a period of 18 days in sea water

The differences between the two microscopic techniques

The glass slides collected from the containers and analyzed under the two microscopy techniques after counting the fields in case of the sea water areas reveled a progressive increase of the cellular density was observed in the case of the biofilms formed in the seawater containers analyzed under bright field microscopy compared to the ones analyzed under epifluorescence microscopy.

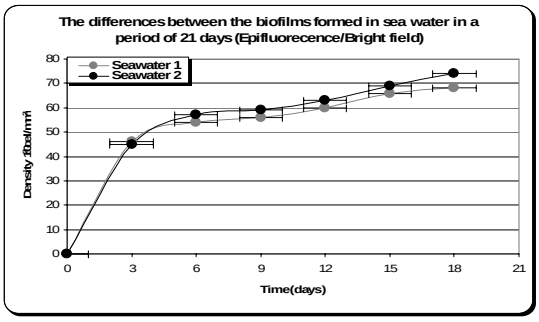


Fig. 6. The differences between the temporal dynamics of the biofilmsfor a period of 18 days in sea water

The glass slides collected from the containers and analyzed under the two microscopy techniques after counting the fields in case of the sediment areas reveled a progressive increase of the cellular density was observed in the case of the biofilms formed in the seawater containers analyzed under epifluorescence microscopy compared to the ones analyzed under bright field (fig. 6). Suci et al. in 1997 makes a series of studies regarding the differed types of microscopy techniques use in the study of bacterial biofilms and recommended the use of epifluorescence and confocal microscopy as les invasive techniques for analyzing and quantifying of adherent bacteria (fig. 7).

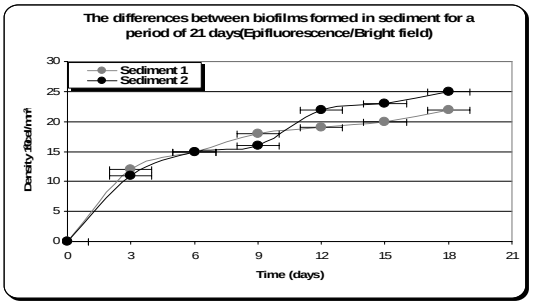


Fig. 7. The differences between the temporal dynamics of the biofilms for a period of 18 days in sea water

In the sediment aria there is a high number of bacteria in the epifluorescence analyses of the slides but after nine days the cell number is higher in the bright field analyses due to the intensive staining of the bacteria cell by simple stains such as the Crystal Violet with can determine errors of quantification.

The values of cellular density obtained in our experiments were lower compared to those obtained by Rijnaarts at al. (1993) 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.

This kind of experiments in static conditions was accomplished "in situ" by Casse and Swan in 2006.

They observed a larger percentage of bacterial density (80%) in static conditions on different types of immersed surfaces in the conditions of marine environment without the application of anti-fouling substances. Some understanding of the ecological conditioning the biofilm may also be drawn from examining the biofilm microorganisms (fig. 8).

Both light and epifluorescence microscopy revealed the presence of many different types of bacterial cells, along with a few eukaryotic cells forms. Kinner et al. in 1993 find that predominant organism in the biofilms examined were filamentous bacterium consisting of rod-shaped. Other filamentous forms were liminated because the contactor bacterium lacked the ultrastructural features of *Cyanobacteria,* endospores, cross-walls and active motility.

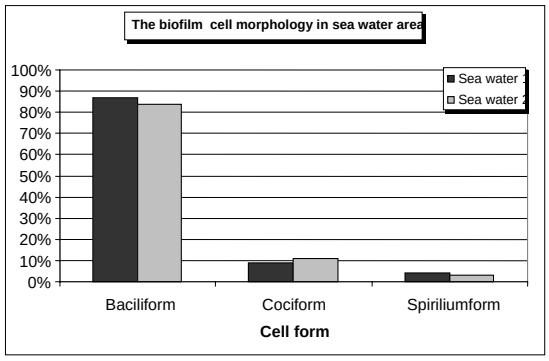


Fig. 8. The differences between biofilms cell morphology in sea water area

Biofilms were formed of a variety of bacteria cell in witch the most common form are bacilli, coci and spirilia. In the slides sea water area bought the microscopy techniques used reveled a high percentage of bacilli of 83 to 87 %, cocci-form between 10% and 12% and spirilia of 3 to 5 % (fig.9).

Biofilms from the first compartments of both contactor pilot plants were extremely dense, forming interwoven mats. These mats were composed of two major constituents: filamentous bacteria, which appeared to be the predominant form, and single-celled bacteria grouped together in amorphous clumps. The latter appeared to be similar to the amorphous film-forming bacteria. Some of the cells in the film-forming bacterial masses also contained these inclusion bodies. (Kinner et al. 1993).

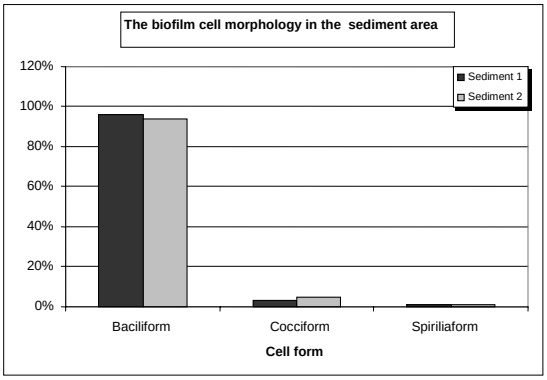


Fig. 9. The differences between biofilms cell morphology in sediment area

In the sediment area the bacilli form all sow have a high percentage of 85 to 89%, the coli form are between 7% and 10% ,and the spirilia have a low percentage between 2% and 4 %.

The cell density in lower that in the sea and interface areas because of the anoxic zone in witch the bacteria have to live, sow only the anaerobe can survive.

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.

At the interface zone there is a thick layer of adherent bacteria do to the nutrient concentration with the highest cell density and percentage of colonization.

The use of epifluorescence microscopy may facilitate the quantification of biofilms density due to the reduction of experimental error frequent in bright field microscopy because of the intense staining.

Epifluorescence microscopy and the use of florochrome helps to differentiate viable and death cell compared cu the bright field microcopy were all the cells and other particles are stained by the simple stain.

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.

Only three days after the immersion of the artificial substrate, a dense layer of cells is formed on the hydrophile surface of glass and 15-18 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 $11 \cdot 10^2$ cel/mm^2 to $74 \cdot 10^2$ cel/mm^2 for the sea water and sediment area separate by the interface.

This information was obtained nondestructively, quasisimultaneously, and in real time, thereby permitting the verification of time-dependent relationships between the biofilms population structure, distribution, and interfacial chemistry. The approach offers opportunities to examine these relationships on a variety of substrata in the presence of a bulk aqueous phase under controlled hydrodynamic conditions.

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