

DOI 10.2478/v10009-010-0012-x
Original research paper

Received: September 14, 2009
Accepted: February 17, 2010

Biofilm development on acrylic coupons during the initial 24 hour period of submersion in a tropical coastal environment

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Key words: biofouling, conditioning film, fouling community, microfouling, Kudankulam

Abstract

The sequence of biofilm formation on a hard surface during the first 24 hours in a coastal environment was studied by suspending acrylic coupons. Adsorption of carbohydrates, proteins, calcium, magnesium, nitrite, nitrate and phosphate were monitored along with microbes. The results showed that carbohydrate, protein, nitrite and nitrate were adsorbed on the coupons within an hour of exposure. Carbohydrates showed a maximum value of 0.28 mg cm⁻² after 24 hours and protein concentration reached up to a maximum of 0.41 mg cm⁻². Adsorption of calcium and magnesium was observed after three hours. Settlement of bacteria was also observed on coupons within an hour and diatoms were observed after 15 hours. Diatoms such as *Navicula* and *Nitzschia* were the dominant colonizers during the early stages of biofilm development.

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INTRODUCTION

When a solid surface is submerged in an aquatic medium, it experiences a series of physical, chemical and biological events leading to the formation of biofilm. In recent years there has been a growing interest in biofilms, owing to their significance in environmental, industrial and medical areas (Donlan 2002). Biofilm formation is the preliminary stage in the process of biofouling on submerged objects in the marine environment (Maki 1999). The presence of fouling on submerged structures can lead to a reduction in their performance, such as damage to static structures and underwater equipment or reduced speed and increased fuel consumption in ships (Evans and Clarkson 1993, Clarkson 1999).

Various steps are involved in the formation of biofilm on submerged surfaces in the aquatic environment. According to Loeb and Neihof (1975), the first step is the adsorption of macromolecules onto the surface. The process is known as conditioning film or molecular film formation. Natural water contains a large number of biomolecules that are the result of breakdown of formerly living material. These substances get quickly adsorbed onto the solid surfaces submerged in water. Macromolecules that adsorb to the submerged marine structures include proteins, glycoproteins, proteoglycons and polysaccharides (Baier 1980, Fletcher and Marshall 1982), and unspecified macromolecules (Zaidi et al. 1984). The conditioned surfaces are then colonized by microbes like bacteria, fungi, microalgae and protozoa. This is the second stage of fouling referred to as microfouling.

Biofilms play a significant role in the biogeochemical cycles and dynamics of coastal ecosystems (Schorer and Eisele 1997). Biofilms may serve to transfer surface water nutrients to the benthos by sedimentation, alter the transfer of carbon and nitrogen to form the dilute pool of dissolved material and provide direct links to higher trophic levels. Due to the accumulation of nutrients, pollutants and their characteristic response to the changes in water quality, biofilms are also widely used in environmental monitoring studies (Gold et al. 2002, Mages et al. 2004).

It has been well established that during the process of biofouling on hard surfaces, the development of biofilm may induce the settlement of larval forms of sessile invertebrates and lower chordates. Biofilms can play an important role in mediating settlement and metamorphosis of invertebrate larvae (Maki et al. 1988). As biofilm formation leads to the settlement of macrofouling communities, studies on the assessment of its structure are a key analytical technique for determining the biofouling potential of a given area.

A clear picture of the fouling process cannot be obtained without considering the initial events in the formation of primary film on the substratum

and its major components, hydrology of the aqueous medium and the types of organisms involved. There are several studies from tropical waters that have characterized the biochemical and molecular constituents to assess the development of biofilm on various hard surfaces (Venugopalan et al. 1994, D'Souza and Bhosle 2003, Bhosle et al. 2005). However, there is a lack of information on biofilm development during the initial 24 hour period. Hence, in the present study an attempt has been made to observe the initial events in biofilm formation on a submerged surface. The objectives of the present study were (1) to examine the events involved in the biofilm development on acrylic coupons exposed to natural seawater during the first 24 hour period and (2) to quantify the major inorganic and organic compounds which accumulated on the surface during the initial hours.

MATERIALS AND METHODS

Acrylic coupons (10×10×0.3 cm) fitted in a wooden frame were suspended at a depth of 1 m at Kudankulam (8° 9' 52'' N and 77° 42' 42'' E) off the East Coast of India during June 2005. Further information on the study area was reported in Satheesh and Wesley (2008). Acrylic was selected as the test material for this study due to its excellent environmental stability compared to other plastics. Before immersion the coupons were washed thoroughly, dried and rinsed with 70% alcohol (Rao 2003). Coupons (in replicates, n=3) were retrieved after 1, 3, 6, 9, 12, 15, 18, 21 and 24 hours of exposure and brought to the laboratory in a box containing filtered (Millipore) seawater. The experiment was replicated (n=4) during the same month and the mean ± standard deviation for a total of 12 coupons (the experiment was replicated four times with three coupons in every observation, 4×3=12) were considered.

In the laboratory, the retrieved coupons were carefully rinsed with filtered seawater (Millipore, 0.45 µm) to remove unattached planktonic forms. The biofilm was scraped off using a sterile nylon brush and the material was dispersed in 50 ml of filtered and sterilized seawater. This biofilm suspension was divided into two parts, one for the study of the microfouling community and the other for biochemical analysis. Filtered and sterilized seawater (blank) was also analyzed for all the relevant biochemical parameters and the values were subtracted from the biofilm values to determine the actual concentration of biofilm. For the estimation of carbohydrate, protein, nitrite, nitrate, phosphate, calcium and magnesium, the biofilm suspension obtained was made up to known volume and filtered through a GF/C membrane filter (0.45, Whatman®) using a Millipore filtering unit.

Total carbohydrate was estimated by phenol-sulphuric acid method (Dubois et al. 1956) using glucose as standard. The protein concentration of the

biofilm was estimated by the method described by Lowry et al. (1951) using bovine serum albumin as standard. For the estimation of nitrate, nitrite and phosphate, the methods given by Venugopalan and Paulpandian (1989) were used. In general, the nitrate was estimated by reducing nitrate to nitrite by passing the water samples through a cadmium column. The cadmium column was prepared using cadmium filings and metallic copper. The nitrite was estimated using the diazotisation method by measuring the extinction at 543 nm. The phosphate was estimated by adding a mixture of reagent containing molybdic acid and trivalent antimony. The resulting blue complex was estimated by measuring the extinction at 810 nm. For the estimation of calcium and magnesium, the samples were completely digested with conc. HNO_3 and H_2O_2 . The digested biofilm sample was diluted at known concentrations with 1N HCl and analyzed by Atomic Absorption Spectrophotometer (Hitachi Z 5000 Polarized Zeeman AAS) using the acetylene–nitrous oxide flame.

For quantitative examination of bacterial colonies, the biofilm sample obtained above was serially diluted (10-fold) using filtered sterilized seawater and 0.1 ml was plated on ZoBell marine agar (Hi Media, India) by pour plate method. The inoculated Petri dishes were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 24 h and the total viable count was noted. Total bacteria count was expressed as CFU cm^{-2} . Qualitative and quantitative analyses of diatoms were done with the help of a binocular microscope. Diatoms were counted using a haemocytometer. For the identification of diatoms, the key provided by Tomas (1997) was used. Correlation analysis was applied to assess the relationship of nutrient concentration with exposure duration.

The hydrological parameters of the coastal waters like water temperature, dissolved oxygen content, salinity, pH, nitrite, nitrate and phosphate were also analyzed during the panel exposure (including replicate experiment period) and the mean values were considered. The surface water temperature was measured *in situ* using a Centigrade thermometer during the early hours (6-6.30 am). For the estimation of dissolved oxygen, water samples were collected in 125 ml BOD bottles and fixed immediately (Winkler's method). Salinity was estimated using a salinity refractometer (ATAGO) and the pH was analyzed using a water quality analyzer (ELICO, India). Nitrate, nitrite and phosphate were estimated according to the methods described by Venugopalan and Paulpandian (1989).

RESULTS AND DISCUSSION

The hydrological parameters of the coastal waters during the study period are given in Table 1. Results showed that carbohydrate, protein, nitrite and nitrate were the microfouling products initially adsorbed on the test coupons. The concentration of carbohydrate after one hour of exposure was

Table 1

Hydrological parameters of the coastal waters during the study period (mean value of four samples collected during June 2005).

Parameters	Values
Salinity	31.8 ±1.3
Temperature	28 ±0.9°C
Dissolved Oxygen	4.68 ±1.08 ml l ⁻¹
pH	7.9 ±0.06
Nitrite	3.9 ±0.08 µg-at N l ⁻¹
Nitrate	3.14 ±0.014 µg-at N l ⁻¹
Phosphate	0.115 ±0.011 µg-at P l ⁻¹

0.002 mg cm⁻² and it showed a maximum of 0.28 mg cm⁻² after 24 hours (Fig. 1). The protein concentration showed a gradual increase from 0.008 mg cm⁻² (after 1 hour) to 0.41 mg cm⁻² (Fig. 1). Protein was more abundant than carbohydrates during the first 24 hours of the exposure period. Nitrate concentration after one hour was 0.003 µg-at N cm⁻² and increased up to a maximum of 0.25 µg-at N cm⁻² after 24 hours (Fig. 2). Nitrite concentration of the biofilm varied between 0.005 (after 1 hour) and 0.095 µg-at N cm⁻² (after 24 hours) during this study period (Fig. 2).

The presence of phosphate, calcium and magnesium were observed on the coupons exposed for three hours duration. Calcium concentration varied between 0.0018 and 0.07 mg cm⁻² (Fig. 3) and the concentration of magnesium varied from 0.004 to 0.031 mg cm⁻² (Fig. 3). Phosphate concentration after three hours of coupon exposure was 0.002 mg cm⁻² and increased up to 0.019 mg cm⁻² after 24 hours (Fig. 4). In general, all the nutrients showed an increasing trend by the end of 24 hours of biofilm growth. This was evident from the significant positive correlation of nutrient concentration with exposure duration (Table 2).

Settlement of bacteria was observed within an hour of coupon exposure and their abundance increased with exposure time (Fig. 5). Diatom settlement was observed on the coupons suspended for a period of 18 hours and *Navicula*, *Nitzschia*, *Pleurosigma* and *Bacillaria* were the genera observed during the study period. *Nitzschia* and *Navicula* showed maximums of 84 ±5.4 and 67.2 ±7.1 cells cm⁻² respectively after 24 hours. The other two genera were not abundant during this period (Table 3).

Results indicated that the process of biofilm development in the study area was quick and took place immediately after the immersion of the coupons. Previous studies on the chemical composition of marine conditioning films

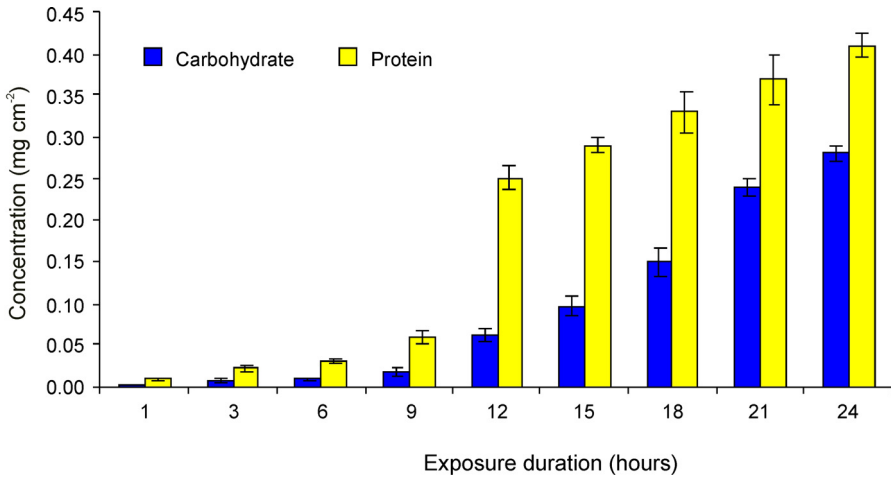


Fig. 1. Concentration of carbohydrate and protein during the initial 24 hours of biofilm development on acrylic coupons (mean ± SD).

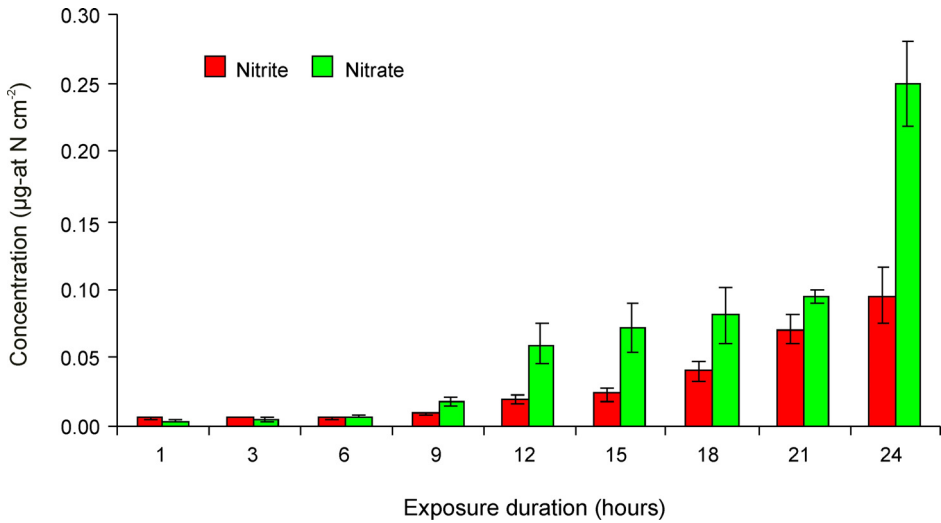


Fig. 2. Changes in the concentration of nitrite and nitrate during the early stages of biofilm development.

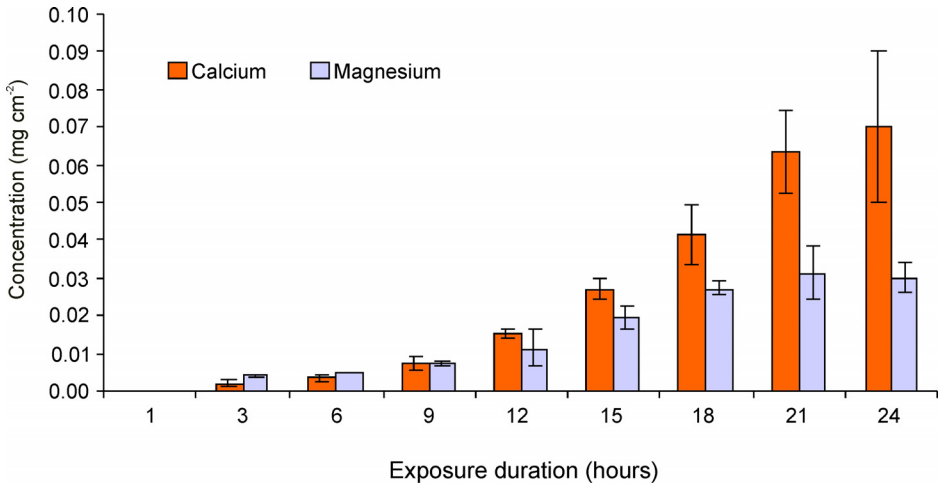


Fig. 3. Changes in the concentration of calcium and magnesium in biofilm developed on acrylic coupons during the initial 24 hour period (mean \pm SD). Calcium and magnesium were observed after 3 hours of exposure.

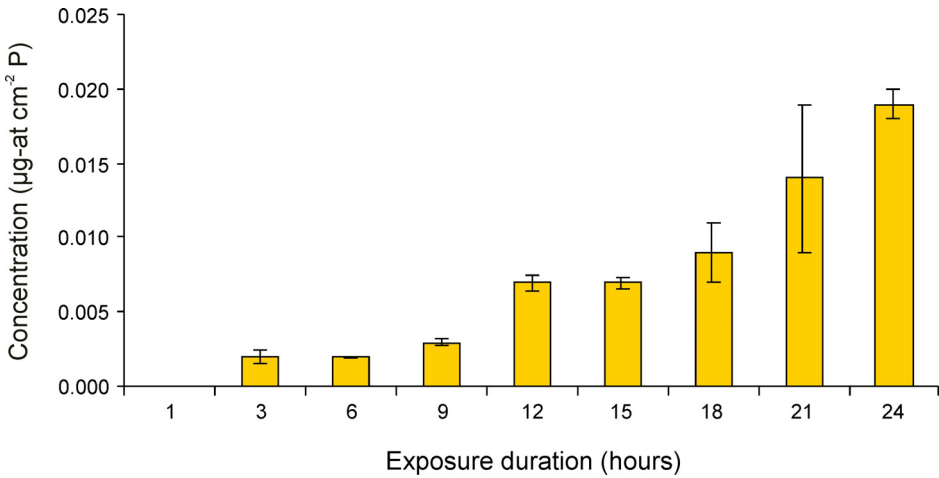


Fig. 4. Concentration of phosphate in biofilm developed on acrylic coupons submerged for a period of 24 hours. Phosphate was observed after 3 hours of exposure.

Table 2

Correlation coefficient of nutrient concentrations against exposure duration (* significant at 5% level).

Nutrients	Correlation coefficient (r)
Carbohydrate	0.94*
Protein	0.96*
Nitrate	0.86*
Nitrite	0.9*
Phosphate	0.95*
Calcium	0.95*
Magnesium	0.97*

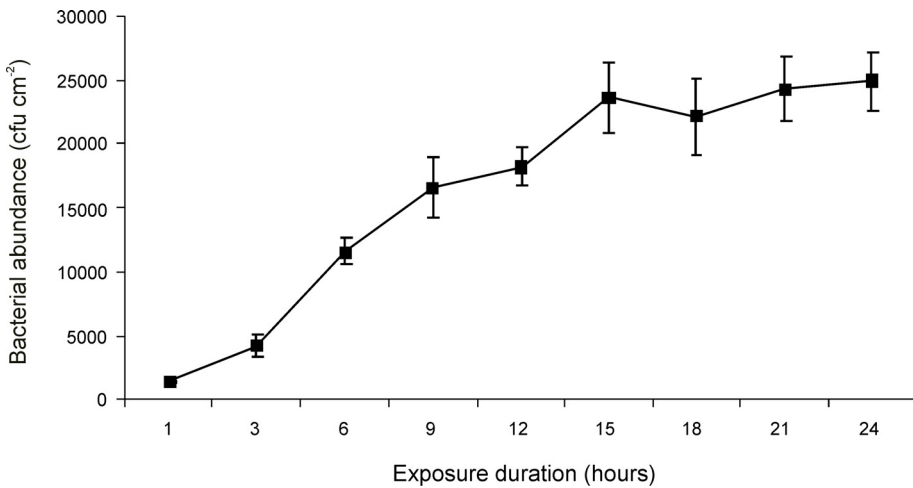


Fig. 5. Settlement pattern of bacteria during the first 24 hours of exposure (mean ±SD).

showed the presence of proteins, nucleic acids, polysaccharides, lipids, humic acids and aromatic acids after three days of exposure (Taylor et al. 1997). Proteins showed higher concentration than carbohydrates during the 24 hour study period. Compere et al. (2001) suggested that proteins were the first to adsorb on the surfaces, followed by carbohydrates. However, D’Souza et al. (2005) reported higher concentrations of carbohydrates in biofilms during the first 5 days of exposure. According to D’ Souza and Bhosle (2003) the carbohydrate and protein concentrations increased with longer exposure times.

Table 3

Colonization pattern of diatoms on test coupons during the first 24 hour period (mean numbers \pm SD cm^{-2}). Numbers in the first row indicate the period of coupon exposure in hours.

Genera	1	3	6	9	12	15	18	21	24
<i>Navicula</i>	0	0	0	0	0	0	0	24.3 \pm 4.6	672 \pm 71
<i>Nitzschia</i>	0	0	0	0	0	0	5.8 \pm 0.9	23.1 \pm 1.9	84 \pm 5.4
<i>Pleurosigma</i>	0	0	0	0	0	0	0	2.3 \pm 0.6	5.5 \pm 1.2
<i>Bacillaria</i>	0	0	0	0	0	0	0	0	1.3 \pm 0.4

In the present study also these compounds showed an increasing trend with time.

The abundance of proteins in biofilms during the initial hours indicates their significance in biofilm development. Several studies have shown that bacteria and phytoplankton populations produce lower concentrations of carbohydrate and higher amounts of protein during the early logarithmic growth phase (Rice et al. 2000, D'Souza et al. 2005). According to Snopok and Kotykevich (2006), the rate of protein adsorption is proportional to the concentration of protein in a solution. Hence, the higher concentration of proteins observed during the initial period may be due to the production of proteins by the biofilm community or from the coastal waters.

Adsorption of all the nutrients selected in this study was observed within three hours of exposure. Nutrients, particularly nitrogen and phosphorous are often considered as limiting factors for the bacteria, fungi and algae in biofilm systems (Tank and Webster 1998, World and Harshey 1999). Extracellular polymeric substance (EPS) production by the microbes in the biofilm is also known to be affected by the nutrient status of the growth medium (Sutherland 2001). The observation of calcium and magnesium after three hours of coupon exposure indicate that these compounds play a key role in biofilm matrix after the microbial settlement. The unique property of the calcium ion is to promote both specific and non-specific interactions with protein and polysaccharide adhesin molecules at the cell surface. Several studies have shown that Ca^{2+} has a significant impact on biofilm development (Koerstgens et al. 2001, Lattner et al. 2003) and the role of Mg^{2+} is largely unknown. The sequential accumulation of calcium and magnesium observed in this study indicated that more studies are required on their role in biofilm formation.

The concentration of compounds in the immersion medium may also influence the adsorption onto surfaces. During the period of study, the concentration of nitrate, nitrite and phosphate were monitored in the surrounding coastal waters. Further, concentration of these nutrients in the water

medium was high when compared to the concentration in the biofilm. Correlation of results obtained from the water samples with that of biofilm is not possible since the present study is conducted for a period of 24 hours duration.

Another important factor within the biofilm matrix which may have a greater influence on the observed variations in the nutrients is the composition and density of the microbial population. A cultivable bacterial population was observed on the test coupons after one hour of exposure. This indicates that bacterial settlement occurred on the coupons within an hour of exposure. Daniel (1963) also reported that bacterial settlement occurred on glass coupons within an hour of exposure at Madras coast (East coast of India). In general, the bacterial population showed an increase in abundance with exposure time. *Nitzschia* and *Navicula* were the dominant diatoms observed during the initial 24 hour period. Previous studies from various parts of the world reported that diatom fouling was mainly dominated by *Navicula*, *Nitzschia* and *Amphora* (Callow 1986, Redekar and Wagh 2000, Patil and Anil 2005). In addition, diatoms play a significant role in the nutrient dynamics of biofilm through syntrophic interactions (Redekar and Wagh 2000).

In conclusion, the observations provide useful information regarding the nutrient cycling in biofilms during the initial 24 hour period in coastal waters. Further, in the present study, only a few chemical and biochemical compounds were monitored and hence it does not imply that only these compounds are involved in the biofilm formation during the first 24 hours. Further studies on the structure and characters of the adsorbed compounds and the identity of bacterial populations are also needed to understand their role in biofilm development.

ACKNOWLEDGEMENT

We thank the Ministry of Earth Sciences (DOD-OSTC), Govt. of India for providing financial assistance.

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