

16. Chemical and Molecular Characterization of Biofilm on Metal Surfaces

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Introduction

Soon after immersion solid surfaces adsorb dissolved organic and inorganic materials onto their surfaces thereby conditioning the surfaces (Loeb and Neihof, 1977; Characklis and Escher, 1988; Taylor et al., 1997). Organic compounds such as glycoproteins (Baier, 1980), humic substances (Loeb and Neihof, 1975), proteins and polysaccharides (Compere et al., 2001) have been implicated as conditioning-film agents. Compere et al (2001) suggested that proteins are the first to adsorb onto surfaces followed by carbohydrates. Conditioned surfaces are then colonized by microorganisms such as bacteria, diatoms, fungi and protozoa apart from the detrital matter derived from marine and terrestrial sources. Thus, the attachment and growth of microorganisms on surfaces is known as biofilm or microfouling (Characklis and Cooksey, 1983).

Microorganisms do not attach to surfaces directly. This is because in aquatic environments both microorganisms, especially bacteria and surfaces generally exhibit a negative charge on their surfaces. In order to avoid this repulsive barrier, microorganisms produce exopolymeric substances and/or other cellular appendages which help the microorganisms to develop contact with surfaces and form biofilm. Exopolymeric substances are generally rich in carbohydrates (Decho, 1990; Welch et al., 1999). Microbial adhesion to surfaces may also induce production of exopolysaccharides (Davies et al., 1993; Vandevivere and Kirchman, 1993) which are useful in biofilm maintenance and protection (Read and Costerton, 1987). The concentration and composition of carbohydrates may vary with microbial species, nutrient availability, growth stage and other environmental parameters (Decho, 1990). Despite their importance in biofilm and microbial corrosion very little is known about the production and characterization of biofilm carbohydrates.

There are several studies describing the biological characterization of biofilms developed on various surfaces (Baier, 1984; Maki et al., 1990; Bott 1993;

Sonak and Bhosle, 1995). As compared to this a few researchers have used molecular approach to assess the development of biofilm (White and Benson, 1984; Khandekar and Johns, 1991; Bhosle and Wagh, 1997). We have used a multi-parameter approach based on biological, chemical, biochemical and molecular constituents to assess development of conditioning film and biofilm on metal surfaces (Bhosle et al., 1989; Bhosle et al., 1990; Sonak and Bhosle, 1995; Bhosle and Wagh, 1997, D'Souza and Bhosle, 2003). This chapter is a compilation of relevant information to understand the dynamics, nature and sources of biofilm organic matter developed on metal surfaces.

Conditioning film

Adsorption of the organic and inorganic matter onto the surfaces immersed in an aquatic environment is the first step in the development of fouling. This is generally defined as the development of the conditioning film or molecular film (Loeb and Neihof, 1977; Characklis and Escher, 1988; Taylor et al., 1997). The adsorbed organic matter is not present as a continuous layer but forms a discontinuous film with variable thickness (Compere et al., 2001). A number of compounds such as glycoproteins (Baier, 1980), humic material (Loeb and Neihof, 1975) and/or unspecified macromolecules (Zaidi et al., 1984) are involved in the development of the conditioning film. Taylor et al. (1997) reported the presence of a number of organic compounds including proteins, lipids, humic acids, nucleic acids, polysaccharides and aromatic amino acids. More recently, Compere et al. (2001) employed several surface analytical instrumental techniques to assess the kinetics and chemical composition of the conditioning film developed on stainless steel panels deployed in seawater. These studies suggest that proteins are the first compounds to adsorb onto stainless steel surface followed by polysaccharides. This supports the earlier reports suggesting the abundance of proteins in the conditioning film (Baier, 1980). The abundance of proteins in the conditioning film was further confirmed by characterizing the conditioning film developed on

stainless steel by the Time of flight secondary ion mass spectrometer (ToF-SIMS) (Poleunis et al., 2002, 2003). Further the nature of the substratum influences the chemical composition and quality of adsorbed organic matter, especially during the initial 4 h period following immersion (Little and Zsolnay, 1985; Taylor et al., 1997). It appears that the procedure used for cleaning the surfaces also influenced the kinetics and the chemical composition of the conditioning film. For example, cleaning the surface with hexane results in slower adsorption of nitrogen containing compounds compared to the surfaces treated with acetone. It is well demonstrated that the conditioning film influence the substratum surface properties including surface charge, wettability and surface free energy which, on the other hand, is known to play an important role in initial colonization of surfaces by microorganisms (Dexter, 1979; Baier, 1980; Fletcher and Marshall, 1982; Schneider, 1997). Therefore a better understanding of the chemical composition and kinetics of the conditioning film formation is essential to explain the biofilm development and biofilm formation by microorganisms such as bacteria, diatoms and fungi.

Characterization of Conditioning-film

Development of conditioning-film on glass cover slips immersed in marine waters over a period of 24 h is shown in Fig. 1. This figure clearly shows the accumulation of organic carbon and nitrogen on glass surface during the 24 h period of immersion in seawater (Bhosle et al., 2004). However, concentrations of the organic carbon in conditioning-film were relatively higher than that of the total nitrogen. Using several surface analytical techniques Compere et al. (2001) reported a similar chemical composition. These observations suggest that the conditioning-film has high C/N (atomic) ratio (Fig. 2) indicating the presence of degraded material or terrestrially derived organic matter. If this is so then the obvious question then is, why bacteria prefer to settle on a surface containing the degraded organic matter?. It is possible that the initial colonizing bacteria have the ability to use this degraded material. Alternatively, microorganisms may prefer to settle on the surfaces due to presence of adsorbed nutrients, which can be used by the colonizing bacteria. Further, it is also possible that the conditioning-film organic matter may modify the physical and chemical properties of the surface that favour the settlement of microorganisms. Nevertheless, the C/N ratio provides conservative evaluation of the nature of organic matter present in conditioning-film. This warrants further studies using

established molecular biomarkers such as amino acids, aldoses and lipid molecules. Such an approach provides useful information on dynamics, sources, nature and degradation state of the organic matter

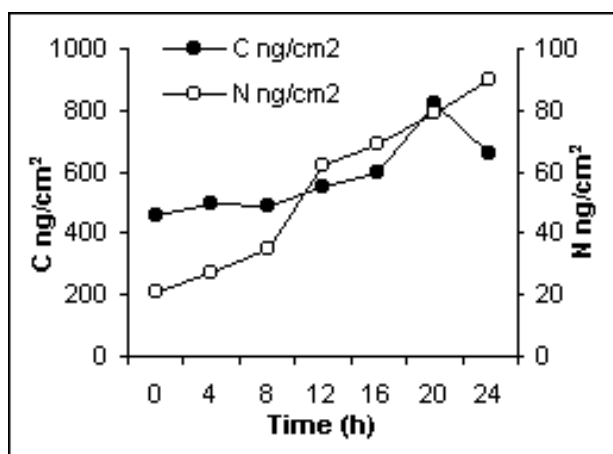


Fig. 1. Changes in organic carbon and organic nitrogen (ng/cm²) of the conditioning film developed on glass panels immersed over a period of 24 h in the surface waters of Dona Paula Bay.

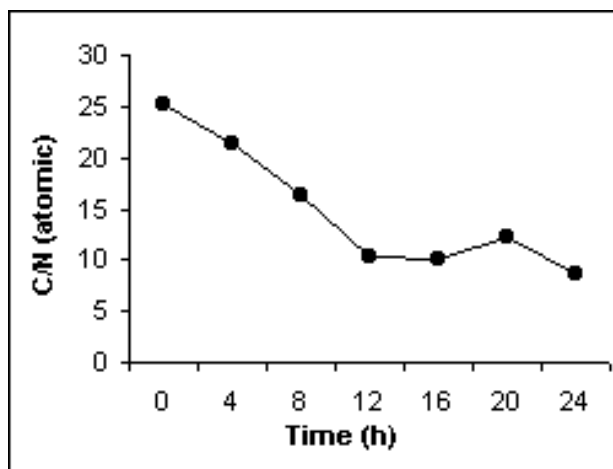


Fig. 2. Changes in C/N ratio (atomic) of the conditioning film developed on glass panels immersed over a period of 24 h in the surface waters of Dona Paula Bay.

(Cowie et al., 1995). Recently Bhosle et al (2004) used a molecular approach based on the total hydrolyzable amino acid (THAA) concentration and composition to assess the nature of the conditioning film organic matter developed on the glass panels immersed in marine waters.

Amino acids in the conditioning film

THAA concentrations of the conditioning film showed small non-linear increase over the period of immersion (Fig.3). THAA-C and THAA-N accounted for 3.7 to 6.7 % and 14 to 29 % of the organic carbon and total nitrogen, respectively of the conditioning film.

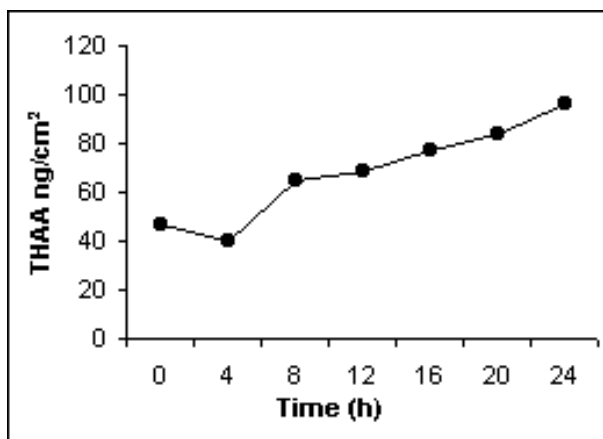


Fig. 3. Changes in THAA (ng/cm²) of the conditioning film developed on glass panels immersed over a period of 24 h in the surface waters of Dona Paula Bay.

Glycine plus threonine (15 to 43 mole %) followed by serine (3.6 to 19.4 mole %), aspartic acid (6.7 to 12 mole %), valine, isoleucine, leucine (2 to 7.5 mole %), lysine, arginine (4.0 to 8.8 mole %), phenylalanine (1.5 to 3.5 mole %) and tyrosine (trace to 1.9 mole %) were the most abundant amino acids in the conditioning-film developed on glass panels. The non-protein amino acid ornithine accounted for trace to 14.7 mole % of the THAA. As the period of immersion increased amino acid composition of the conditioning film changed (Fig. 4). During the first few hours of immersion, glycine plus threonine, serine and ornithine were the most abundant amino acids in the conditioning film developed on glass panels. As the

period of immersion increased the mole % contribution of these amino acids to the THAA decreased. As the period of immersion increased the decrease in glycine plus threonine, serine and ornithine was associated with the increase in mole % contribution of aspartic acid, glutamic acid, phenylalanine, isoleucine, leucine and valine to the total THAA. It has been reported that during degradation of organic matter glutamic acid, tyrosine, phenylalanine, valine, isoleucine and leucine decreased while glycine, threonine and serine generally increased due to their diagenetic behaviour and speciation (Dauwe and Middelburg, 1998). If this is so, then the observed higher mole % contribution of glycine plus threonine and serine to the total THAA during the initial 0 to 8 h period of the conditioning film formation indicates accumulation of degraded refractory organic matter in the conditioning-film. Conversely, the relatively increasing mole % contribution of phenylalanine, valine, isoleucine and leucine suggest its recent biogenic origin. Selective preservation of glycine, serine and threonine has been observed in suspended and sedimenting particulate matter and sediments (Siezen and Mauge, 1978, Lee and Cronin, 1984; Muller et al., 1986). The indicative value of glycine for the refractory nature of organic matter has been reported earlier for dissolved organic matter of seawater samples (Yamashita and Tanoue, 2003). Since these amino acids are enriched in the cell-wall proteins of diatoms, it has been hypothesized that such selective preservation may result from the refractory nature of the protein-silica complex of diatom cell walls.

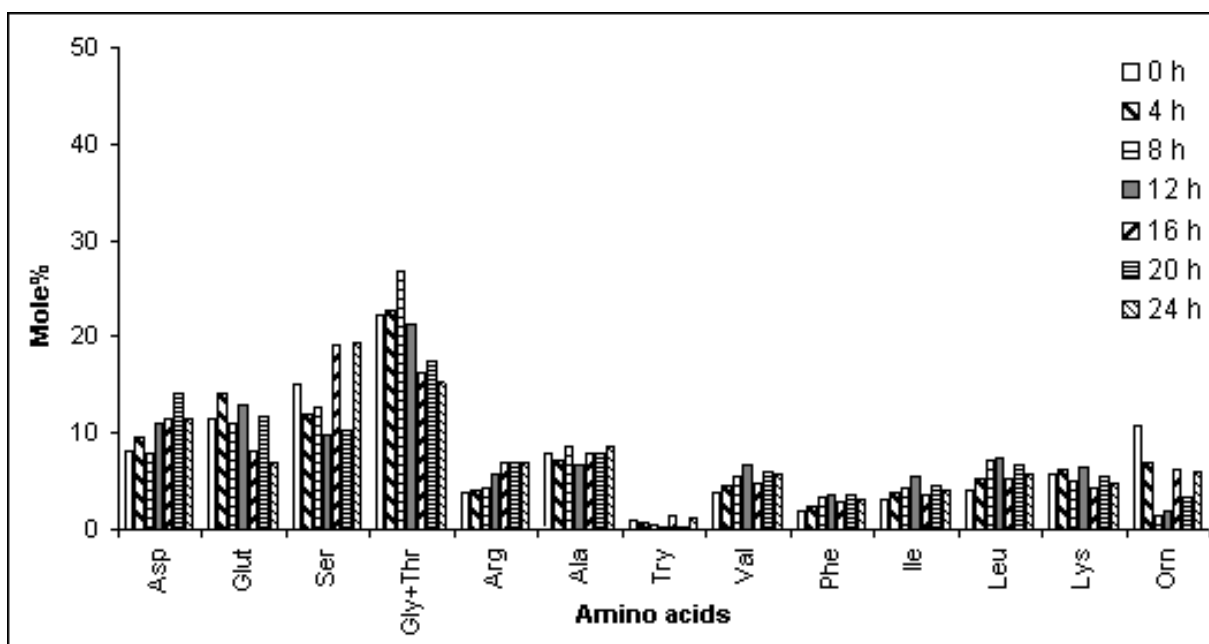


Fig. 4. Changes in the THAA (mole%) compositions of the conditioning film developed on glass panels immersed over a period of 24 h in the surface waters of Dona Paula Bay. Compound abbreviations: Asp = aspartic acid; Glut = glutamic acid; Ser = serine; Gly+Thr = glycine+threonine; Arg = arginine; Ala = alanine; Tyr = tyrosine; Val = valine; Phe = phenylalanine; Ile = isoleucine; Leu = leucine; Lys = lysine; Orn = ornithine.

Biofilm development

A number of parameters such as dry weight, organic carbon (OC), total nitrogen (TN), chlorophyll a and proteins have been used to assess the development of biofilm on various surfaces. Concentration of these compounds of the biofilm material generally increased over the period of immersion (Fig. 5). This implies production of such compounds by the organisms in biofilm. The observed increase was not due to increase in settlement but due to growth of the attached organisms. This was evident from the increase in chlorophyll α concentration of the biofilm samples. The nature of the surface influenced the development of biofilm. Microfouling biomass was higher on nontoxic surfaces such as mild steel and stainless steel, while it was relatively lower on toxic

surfaces such as copper. As observed for the biofilm biomass, the concentration of total carbohydrates (TCHO) in the biofilm samples also increased over the period of immersion.

C/N ratio of the biofilm

C/N ratio has been widely used as a proxy to identify the sources and the nature of organic matter and also to assess the extent of degradation. The rationale behind this approach is that the organic matter derived from fresh microorganisms is relatively rich in proteins thus, has an atomic C/N ratio in the range of 5 to 8, whereas the organic matter of vascular land plants, being rich in cellulose, exhibit C/N ratio of 20 and above (Meyers, 1994). During the process of degradation, nitrogen is preferentially lost relative to organic carbon. This results in increase in C/N ratio.

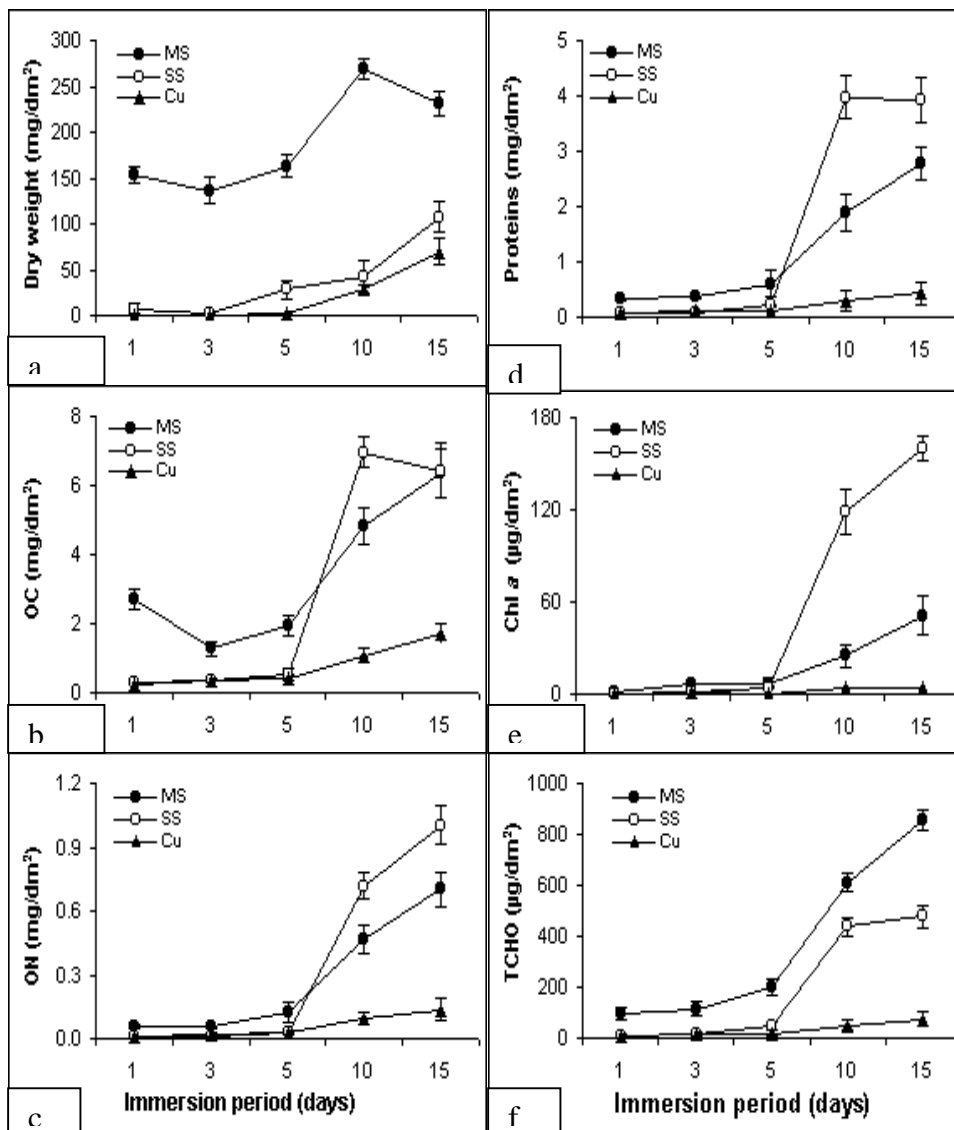


Fig. 5. Changes in biofilm biomass as DW (a), organic carbon (b), organic nitrogen (c), algal biomass as Chl a (d) and protein (e) and the concentration of total carbohydrate of the biofilm (f) on mild steel (?), stainless steel (?) and copper (?) panels immersed in the surface waters of Dona Paula Bay over a period of 15 d. Bar = mean \pm standard deviation.

Biofilm material developed on mild steel, stainless steel, copper and titanium was analysed for carbon and nitrogen so as to assess the changes in C/N ratio of the biofilm. It appears that biofilm developed on these surfaces during the first 3 d period of immersion had relatively high C/N ratio irrespective to the surfaces used (Fig. 6a). As the period of immersion increased the C/N ratio generally decreased. At day 15 following immersion, it decreased to ~7 to 8. This observed low C/N ratio compares well with those reported for laboratory-grown microalgae (Verity et al., 1992; Otero et al., 1998). Thus, C/N ratio provided useful information to understand the abundance of fresh organic matter and its sources. The surface, however, did not influence C/N ratio.

OC/Chlorophyll *a* ratio of the biofilm

The low C/N ratio is useful to know the abundance of fresh organic matter derived from both auto and hetero-trophic organisms. However, the ratio cannot differentiate the relative abundance of auto-trophic or hetero-trophic organisms. Such differentiation can be achieved by using organic carbon/chlorophyll *a* ratio.

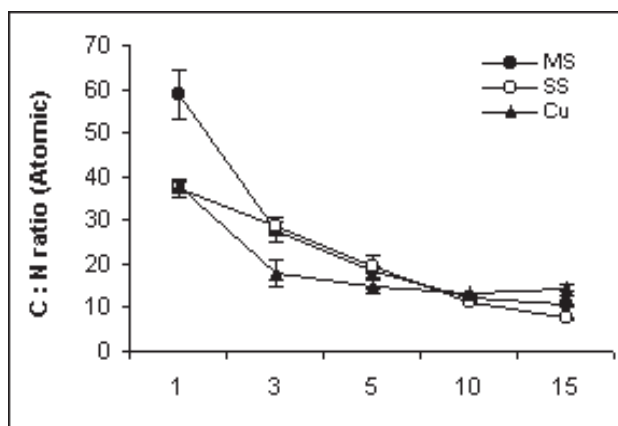


Fig. 6a. Changes in C/N ratio (atomic) of the biofilm on different panels immersed in the surface waters of Dona Paula Bay over a period of 15 d. Bar = mean \pm standard deviation.

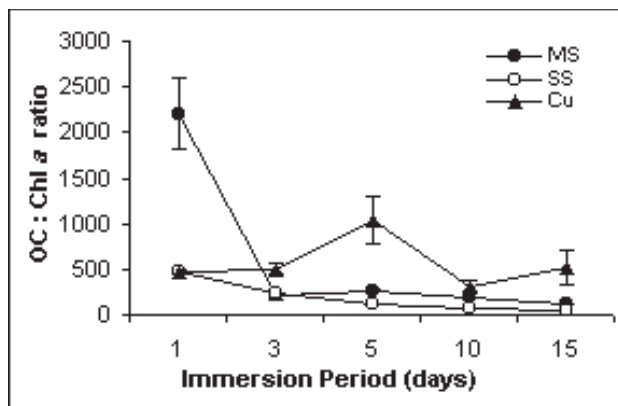


Fig. 6b. Changes in OC:Chl *a* ratio of the biofilm on different panels immersed in the surface waters of Dona Paula Bay over a period of 15 d. Bar = mean \pm standard deviation.

The rationale for this approach is that chlorophyll *a* is very abundant in autotrophic organisms. In actively growing microalgae the ratio is low (< 30). Thus when the algal contribution to biofilm is high the carbon/chlorophyll *a* should decrease to a low value. As observed for the C/N ratio, the organic carbon/chlorophyll *a* ratio of the biofilm developed on various surfaces generally decreased (Fig.6b). The organic carbon/chlorophyll *a* ratio was lowest (30) at days 5 and 10 following immersion. This decrease in organic carbon/chlorophyll *a* and C/N ratio suggests the abundance of microalgal organic matter in the biofilm developed on these surfaces. The observed pattern of decrease in organic carbon/chlorophyll *a* ratio of the biofilm as function of immersion period was similar on toxic and nontoxic surfaces. This implies that irrespective of the type of surface the organic carbon/chlorophyll *a* ratio of the biofilm generally decreased over the period of immersion.

Biochemical characterization of the biofilm

Carbohydrates, proteins, lipids, nucleic acids are generally the most abundant biochemical compounds in biofilm and, their concentrations generally increase with the period of immersion. The relative abundance of these compounds can vary with nature of surfaces. For example, D'Souza and Bhosle (2003) reported increasing concentrations of proteins and carbohydrates to the biofilm organic matter with the increase in the immersion period (Fig. 5e & f). Similarly, they also observed relative differences in the distribution of carbohydrates and proteins in biofilm developed on mild steel (MS), stainless steel (SS) and copper (CU) panels immersed in surface seawater of the Dona Paula Bay (Fig.7a & b). Together, carbohydrate- carbon and protein-carbon accounted for 7 to 31 %, 11 to 31 % and 11 to 14 %, in the biofilm developed on MS, SS and CU, respectively (Fig. 7c).

The concentrations of both carbohydrates and proteins of the biofilm on MS, SS and Cu generally increased over the period of immersion suggesting the production of these compounds by biofilm (Fig. 5e & f). Nevertheless, their relative distribution varied over the period of immersion. Contrary to the general belief, proteins generally were relatively more abundant than carbohydrates in the biofilm for the entire period of immersion (Fig. 5e&f). Laboratory grown diatom cultures, bacteria and natural phytoplankton populations are known to produce lower amounts of carbohydrates and higher amounts of proteins during the early logarithmic growth phase (Myklestad, 1977; Hitchcock, 1978), whilst diatom cells in stationary growth phase produce higher amounts of carbohydrates and lower amounts of proteins.

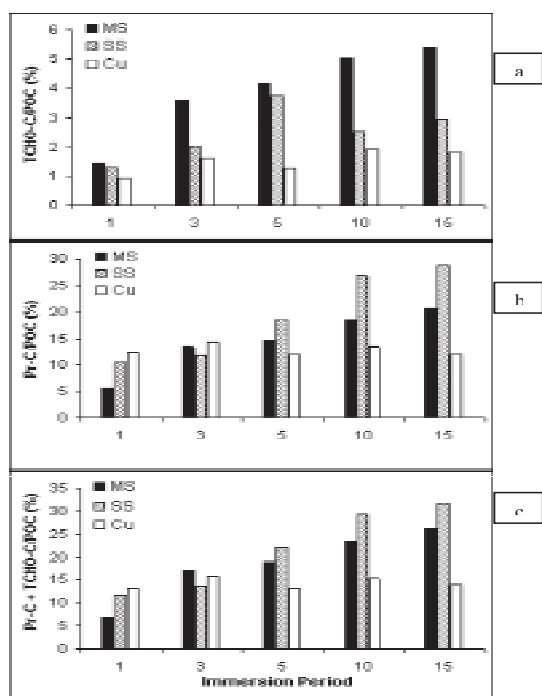


Fig. 7. Average variation in the contribution of carbohydrate-carbon and protein-carbon to organic carbon of the biofilm on different panels, over the 15 d period of immersion in the surface waters of Dona Paula Bay.

In view of this, the observed distribution pattern of carbohydrates and proteins in biofilm on the surfaces probably indicates the effect of growth phase of biofilm organisms. Concentrations of nutrients, especially nitrate and phosphates in the growth medium are known to influence the production of carbohydrates and proteins in the laboratory grown microorganisms. It is well documented that microorganisms, particularly microalgae produce large amounts of carbohydrates and lower amounts of proteins under nutrient-deficient conditions. Chlorophyll α concentrations and microscopic observations suggest the abundance of microalgae in the biofilm developed on MS, SS and Cu. Although microalgae were abundant in the biofilm on MS, SS, Cu, the production of carbohydrates by biofilm microalgae was relatively very low compared to proteins. As nitrate and phosphate concentration in surface seawater generally increased during the period of study, observed trend in the relative production of carbohydrates and proteins by the biofilm organisms probably suggests the effect of nutrients in the surrounding seawater and/or within the biofilm matrix.

Is carbohydrate production induced in biofilm?

Microscopic observations have shown that organisms in biofilm produce large amounts of carbohydrates, especially exopolysaccharides while attaching to

surfaces (Decho, 1990). However, there are a few studies confirming these observations. For example, Vandevivere and Kirchman (1993) reported induced production of carbohydrates by the attached microorganisms. D'Souza and Bhosle (2003) observed microalgal abundance on metal surfaces immersed in natural seawater. From the latter studies, it was seen that contribution of carbohydrate-C to the total carbon of the biofilm varied from 1 to 5 % which means carbohydrates were not the most abundant constituents. Biofilms grown on MS, SS and Cu panels immersed in the natural seawater did not show the abundance of carbohydrates but were relatively enriched in proteins (D'Souza and Bhosle, 2003). Although carbohydrates are important in biofilm formation, studies carried out in our laboratory on natural biofilm suggest the abundance of other constituents such as proteins in microbial biofilms.

Monosaccharide-composition of biofilm

Carbohydrates are among the most abundant constituents in both terrestrial and marine organisms. Carbohydrate polymers are generally present as storage and structural components. Of these, storage polysaccharides are preferentially utilized by heterotrophic organisms and by respiring autotrophic organisms. Such selective removal results in the enrichment of structural polysaccharides in degraded material. Structural polysaccharides retain the original signature of source material. Therefore, composition of monosaccharides provides useful information on the sources of organic matter in an environment (Cowie and Hodges, 1984; Ittekkot and Arain, 1986). For example, mannose: xylose ratio, the total percentage of ribose, ribose plus fucose as well as arabinose plus galactose in sugar fraction (calculated on glucose free basis) have been used to distinguish plant material, bacteria, microalgae and zooplankton sources in various samples (Cowie and Hodges, 1984). Organic matter input from calcareous and siliceous sources can be distinguished using arabinose:fucose ratio (Ittekkot et al., 1984). Similarly, hexoses: pentoses (W:W) ratio can be used to assess the presence of soil organic matter derived from terrestrial plant (Stevenson, 1982; Ittekkot and Arain, 1986). The rationale behind this approach is that the terrestrially derived soil organic matter is rich in pentoses compared to hexoses thus giving low hexoses: pentoses (W:W) ratio. Biofilm material developed on MS, SS and Cu was analysed for monosaccharide composition. Capillary GC analysis of biofilm material showed the presence of glucose, galactose, mannose, xylose, arabinose, ribose, rhamnose and fucose. The concentrations of these monosaccharides of the biofilm developed on all the test surfaces generally increased suggesting the

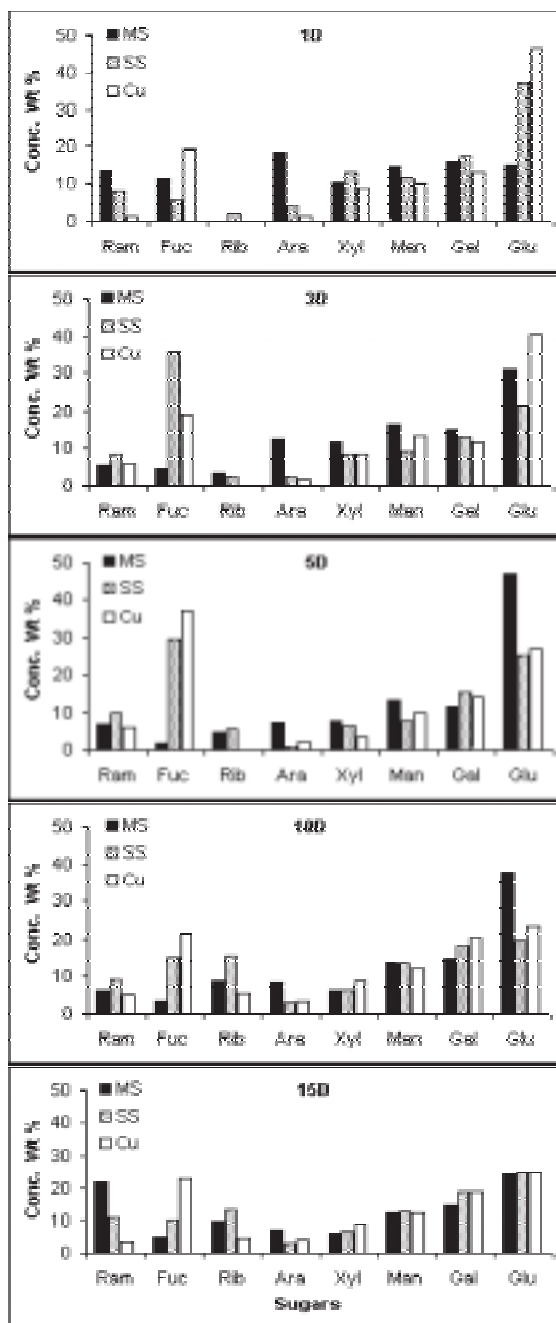


Fig. 8. Average monosaccharide composition biofilm developed on different panels over the 15 d period of immersion in the surface waters of Dona Paula Bay.

production of these compounds by the biofilm organisms over the period of immersion. Nevertheless, the contribution of individual monosaccharides to the total carbohydrates of the biofilm varied over the period of immersion as well as with the type of surface (Fig. 8).

At day 1 of the immersion arabinose was most abundant followed by galactose, glucose, rhamnose, fucose and xylose, whereas ribose could not be detected in the biofilm developed on Ms panels (Fig. 8). With the increase in the immersion period, the wt

percent contribution to total carbohydrates (sum of all the identified monosaccharides) of arabinose, fucose, xylose, mannose, galactose and rhamnose decreased while that of ribose and glucose increased. At day 15 following immersion glucose (24 wt %), rhamnose (22 wt %) and ribose (9 wt %) were most abundant monosaccharides in biofilm developed on MS panels. Higher wt percent of rhamnose may indicate the presence of bacteria. As ribose was not detected in this sample bacterial biomass was not probably the major contributor to the elevated levels of rhamnose. Relatively higher levels of rhamnose plus fucose accompanied by lower levels of glucose were recorded for the biofilm developed on MS at day 1 following immersion. These monosaccharides demonstrated similar distribution trends for the more degraded riverine particulate matter and vascular plant tissues (Hedges et al., 1994; Opsahl and Benner, 1999). Therefore, the observed higher abundance of rhamnose plus fucose associated with lower abundance of glucose may indicate the presence of degraded terrestrial material in the biofilm developed on MS at day 1 following immersion. This conclusion was further supported by the abundance of arabinose (18 %) and xylose (10 %) in the biofilm on MS at day 1 of the immersion period. Both these monosaccharides do not contribute significantly to whole cell or the cell wall material of diatoms and bacteria but are abundant in terrestrial plants as constituents of arabinoxylan and arabinoglucuronoxylan (Cowie and Hedges, 1984). Thus, data on monosaccharides composition strongly indicates the presence of degraded terrestrial material in the biofilm on MS at day 1 of the immersion period.

As the period of immersion increased the wt % abundance of rhamnose, fucose, arabinose and xylose decreased while that of glucose increased from 15 to 47 %. At day 5, the monosaccharides composition of the biofilm is similar to that observed for natural phytoplankton, suggesting the abundance of micro-algal material on MS (Cowie and Hedges, 1984). This was also evident from high abundance of hexoses compared to pentoses (Fig. 9). Hexoses are relatively more abundant in microalgae while pentoses are abundant in bacteria (Khandekar and Johns, 1990). Between days 5 and 15 of the immersion the glucose concentration decreased again (from 47 to 24 %) suggesting yet another change in the biofilm community structure (Fig. 8). This decrease was associated with relative increase in rhamnose (22 %) and ribose (9 %). Hicks et al (1994) observed abundance of both these monosaccharides in bacteria. For example, rhamnose was relatively abundant in *Pseudomonas* sp, *Azotobacter* sp and *Caulobacter* sp. D'Souza and Bhosle (2003b) reported the presence of these bacteria on the MS panels immersed in the surface waters of the same study

site. Moreover, Cowie and Hedges (1984) also reported the abundance (11 to 60 %) of ribose in some unidentified species of bacteria. D'Souza (2004) analysed the monosaccharides composition of some unidentified species of bacteria in which the ribose accounted for 10 to 30 % of the total carbohydrates. Therefore, relatively higher contribution of rhamnose (22 %) and ribose (9 %) to total carbohydrates of biofilm at day 15 of the immersion period may indicate the abundance of bacteria (Fig. 8). Yet another evidence for the abundance bacteria can be obtained from the hexoses and pentoses content of the biofilm on day 15 of the immersion period. Hexoses contribution to total carbohydrates decreased while that of pentoses increased in biofilm at day 15 of the immersion period (Fig. 9). This is consistent with the observation that pentoses are abundant in heterotrophic bacteria.

It is possible that the observed decrease in glucose may also reflect preferential utilization of glucose rich polymers by heterotrophic bacteria. In addition to consumption by heterotrophic bacteria, this removal of glucose may partly be due to the producers themselves. For example, in diatom glucose occurs as β 1,3 glucan, a storage polysaccharide that is easily consumed during their time of survival and respiration. The overall trends in monosaccharides composition of biofilm developed on MS strongly suggest that deposition of degraded material of terrestrial origin was the first step, As the period of immersion increased the degraded terrestrial material was replaced by microalgae. By day 5 of the immersion period, microalgae was abundant in biofilm. By day 15, microalgae were replaced by bacteria. Thus, the monosaccharides composition data provided a better picture of biofilm development on MS that was otherwise impossible to obtain using only microscopic observations.

Monosaccharides composition of biofilm on SS and Cu provided a contrasting picture of biofilm development. There was no evidence of the presence of the degraded terrestrial material in the biofilm developed on these surfaces at day 1 following immersion. Similarly, ribose contribution to the total carbohydrates is too low to suggest the abundance of bacteria. The overall monosaccharides composition data are in the range generally recorded for microalgae (Cowie and Hedges, 1984). The monosaccharides composition appears to be characteristic of a microalgal source, with relatively low percentages of pentoses and high percentages of hexoses. For day 1 to day 10 of the immersion period, glucose in biofilm on SS and Cu decreased (from 46 to 37 % and 23 to 19, respectively). This decrease is associated with an increase in other monosaccharides, especially fucose. Glucose-rich

polymers are replaced by fucose rich storage polymers in some diatoms (Percival, 1970). Fucose is also one of the abundant constituent monosaccharides of the cell walls of *Skeletonema*, *Thalassiosira* and *Nitzschia* species. Generally, these diatoms are abundant in biofilm developed on SS (D'Souza, 2004). Fucose concentration subsequently decreased, and this was followed by an increase in ribose (15 %) and rhamnose (10 %). A higher abundance of ribose (15 %) and rhamnose (10 %) indicates an initiation of a process of replacement of microalgae by bacteria.

Nature of biofilm organic matter

The contribution of carbohydrate-C plus protein-C to the organic carbon is useful to assess the nature of organic matter (Cowie et al., 1995). They both account for 30 to 70 % of organic matter in marine and terrestrial sources. As the degradation proceeds, the content of these compounds generally decrease (Hedges et al., 1994; Pantoja and Lee, 1999). Therefore, the relative contribution of these compounds to total organic carbon indicates the extent to which the degradation of organic matter has taken place. In a study, as the period of immersion increased the contribution of carbohydrate-C plus protein-C to the total organic carbon of the biofilm on MS and SS increased (7 to 26 % and 11 to 31 % organic carbon of biofilm on MS and SS, respectively), whereas it did not vary much (13 to 15 % of organic carbon) in the biofilm on Cu. As their contribution to the organic carbon increased with time, it might imply relative abundance of fresh biogenic organic matter. On the other hand, the contribution of these compounds to organic carbon of the biofilm on Cu did not change much (13 to 15 % of the biofilm organic carbon) throughout the period of immersion, suggesting a higher abundance of degraded material and/or very little growth of biofilm organisms on this toxic surface. Overall, only 7 to 30 % of organic carbon was accounted for these compounds. That means 30 to 93 % of the organic matter of the biofilm remained uncharacterized. Although lipids may have accounted for some of the uncharacterized organic material, a substantial portion of the organic matter of the biofilm remained uncharacterized. It is possible that this uncharacterized organic material may play an important role in subsequent process of biofouling development.

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