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Seasonal variation in the chemical composition and carbohydrate signature compounds of biofilm

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ABSTRACT: Biofilm developed on stainless steel was characterised using biological, chemical and biochemical parameters, as well as aldose molecular biomarkers. Biofilm biomass and carbohydrate concentration increased on stainless steel, whereas C:N and organic carbon:chlorophyll *a* ratios decreased over the period of immersion. Despite the abundance of microalgal biomass, carbohydrate concentration was lower than that observed for proteins. Carbohydrate composition varied during the period of immersion. Glucose, arabinose and xylose were relatively more abundant during the initial period (5 d) of immersion, whereas rhamnose, fucose, ribose and galactose were more abundant during the latter period (>5 d) of immersion. The sugar distribution trends suggest that biofilm carbohydrates were mostly derived from degraded biogenic and/or terrestrial sources, especially during the initial period (<5 d) of immersion. As the period of immersion increased, the contribution of biogenic sources to the biofilm carbohydrates increased. This conclusion was also supported by principal component analysis based on wt % aldose composition. Multi-parameter approaches such as the one used in the present study provide a better picture of the sources and nature of biofilm organic matter.

KEY WORDS: Biofilm biomass · Carbohydrates · Aldose · Organic matter · Stainless steel

INTRODUCTION

Surfaces immersed in an aquatic environment rapidly adsorb dissolved organic matter, thereby conditioning the surface (Loeb & Neiholf 1977, Characklis & Escher 1988, Taylor et al. 1997, Compere et al. 2001). Conditioned surfaces are then colonised by microorganisms. Such attachment and growth of microorganisms on surfaces is generally defined as biofilm (Characklis & Cooksey 1983, Decho 2000). While attaching to surfaces, microorganisms produce extracellular polymeric substances (EPS), generally rich in carbohydrate polymers (Decho 1990, Welch et al. 1999, Wingender et al. 1999). These carbohydrate polymers play an important role in the maintenance and protection of biofilm (Costerton et al. 1987, Read & Costerton 1987, Wolfaardt et al. 1999).

Carbohydrates of organisms have been classified as storage and structural polysaccharides. Storage polysaccharides (labile sugars) are rapidly utilised by *in situ* organisms (Handa & Tominaga 1969, Ittekkot et al. 1982, Tanoue & Handa 1987, D'Souza & Bhosle 2001). Such selective utilisation of storage polysaccharides results in the accumulation of relatively less degradable structural polysaccharides (refractory sugars) (Cowie & Hedges 1992, Burdige et al. 2000, Ogier et al. 2001). Structural polysaccharides are relatively more refractory in nature, and so they are likely to leave a signature of the original source material in the sample. Therefore, aldoses, the monomeric units of carbohydrates, are often used as useful tools to distinguish inputs from terrestrial and marine sources in aquatic environments (Cowie & Hedges 1984).

Most previous studies on biofilm have focused on the microscopic biological characterisation and/or the analysis of the bulk chemical compounds, such as organic carbon and nitrogen (Baier 1984, Bhosle et al. 1989, Maki et al. 1990, Bott 1993, Sonak & Bhosle 1995, D'Souza & Bhosle 2003a). Conversely, little is known, on a molecular level, concerning the biochemical characteristics of biofilms that develop on surfaces immersed in seawater (White & Benson 1984,

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Khandekar & Johns 1990, Bhosle & Wagh 1997, D'Souza & Bhosle 2003b). Such studies on detailed molecular composition, nature and short-term temporal variation would help in understanding the dynamics, origin and nutritional value of the biofilm organic matter (OM) (Hedges et al. 1994). Moreover, increased knowledge of the chemical composition on a molecular level and the kinetics of the biofilm OM is central to a sound scientific understanding of biofilm growth and adhesion mechanisms, as well as to assessments of the effect of specific organic molecules on the settlement of invertebrate larvae (Wieczorek et al. 1995).

The aims of the present study were: (1) to assess the seasonal variations in biofilm carbohydrates over the initial 15 d immersion period, (2) to identify the sources of biofilm carbohydrates and (3) to assess the nature of the biofilm OM over the period of immersion.

MATERIALS AND METHODS

Test panels and deployment. Twenty stainless steel (SS) panels ($25 \times 20 \times 0.05$ cm) were cleaned, sterilised and deployed in the surface waters (~2 m) of Dona Paula Bay (15.31° N, 73.59° E) following procedures described earlier (Sonak & Bhosle 1995, Bhosle & Wagh 1997, D'Souza & Bhosle 2003b). Panels were deployed in April/May 1999, April/May 2000, September 2000 and January 2001. The panels (4) were retrieved on Days 1, 3, 5, 10 and 15 following immersion.

Collection of samples. After retrieval, biofilm material from the test panels was removed using a nylon brush and filter-sterilised seawater (0.2 µm; Nuclepore) (Sharma et al. 1990, Sonak & Bhosle 1995, Bhosle & Wagh 1997, D'Souza & Bhosle 2003b). The scraped biofilm material was collected to a known volume. A known aliquot was filtered through pre-ignited (450°C, 3 h) GF/F filters (47 mm, 0.7 µm pore size) for the analysis of organic carbon (OC), organic nitrogen (ON), chlorophyll *a* (chl *a*), proteins, total carbohydrate ($TCHO_{SP}$) and aldoses. For bacterial counts, 0.5 to 1 ml of the scraped material was fixed with filter-sterilised (0.2 µm filter) 2% formaldehyde and then stored in a refrigerator at 4°C until analysis. Chl *a* samples were processed immediately, whereas the filters employed for all other parameters were stored at –20°C until analysis. All the samples were analysed in triplicate.

Analysis of samples. Chl *a* was analysed by spectrophotometry (Parsons et al. 1984). The OC was estimated following the method of Parsons et al. (1984). The ON was quantified using the method of Smart et al. (1983). Proteins were analysed by the bicinchonic acid method (Smith et al. 1985). $TCHO_{SP}$ was analysed using the phenol-sulphuric acid method (Dubois et al. 1956). The precision of the analytical methods based on 3 replicates was better than 6%.

To estimate total bacterial counts (TBC) a known volume (0.2 ml) of the sample was stained with 0.1% acridine orange and filtered through 0.2 µm Nucleopore filters. Bacteria were counted in 10 randomly selected fields using an epifluorescence microscope (Olympus CX40), as suggested by Parsons et al. (1984).

Aldose composition of the biofilm and the probable sources such as fouling bacteria, diatoms (*Navicula* sp. and *Amphora* sp.), macroalgae (*Gracilaria* sp. and *Sphacelaria furcigera*) and mangrove leaves (*Kandelia* sp. and *Rhizophora* sp.) were determined using the capillary gas chromatographic (GC) technique (Bhosle et al. 1995, D'Souza et al. 2003). In the present study, total aldoses have been defined as the sum of all identified aldoses. The contribution of an individual aldose to the total aldoses is expressed as a percentage of the total weight (wt %). Analytical reproducibility of the GC method was greater than ±6%, whereas it was higher than ±10% for the GC plus sample preparation method.

Statistical analysis. A simple regression analysis was employed to assess the relationship between various parameters using the Excel program on a personal computer (Sokal & Rohlf 1981). A 2-way analysis of variance (ANOVA) was carried out to determine if there were significant daily and seasonal variations in biofilm development. Principal component analysis (PCA) based on wt % aldose composition was performed following the procedure described earlier (D'Souza & Bhosle 2001, D'Souza et al. 2003).

RESULTS

Biomass, chemical and biochemical composition

The biofilm biomass on SS (measured as OC, ON, proteins, chl *a* and TBC) showed small changes over the first 5 d period of immersion (Fig. 1). Thereafter, the biofilm biomass increased over the subsequent period of immersion: values were higher in September 2000 (monsoon) compared to those recorded in January 2001 (post-monsoon), whereas intermediate values were recorded for both the pre-monsoon seasons (April/May 1999 and 2000) (Fig. 1). The 2-way ANOVA of the biomass parameters showed significant daily variations ($F = 8.48$ to 171.20; $p > 0.001$); however, the seasonal variations were not significant ($F = 0.75$ to 17.88; $p > 0.01$ to 0.50), except for TBC ($F = 17.88$; $p > 0.001$). Highly significant positive correlations were observed between various biomass parameters (Table 1). Furthermore, carbohydrates and proteins showed significant positive relationships with both

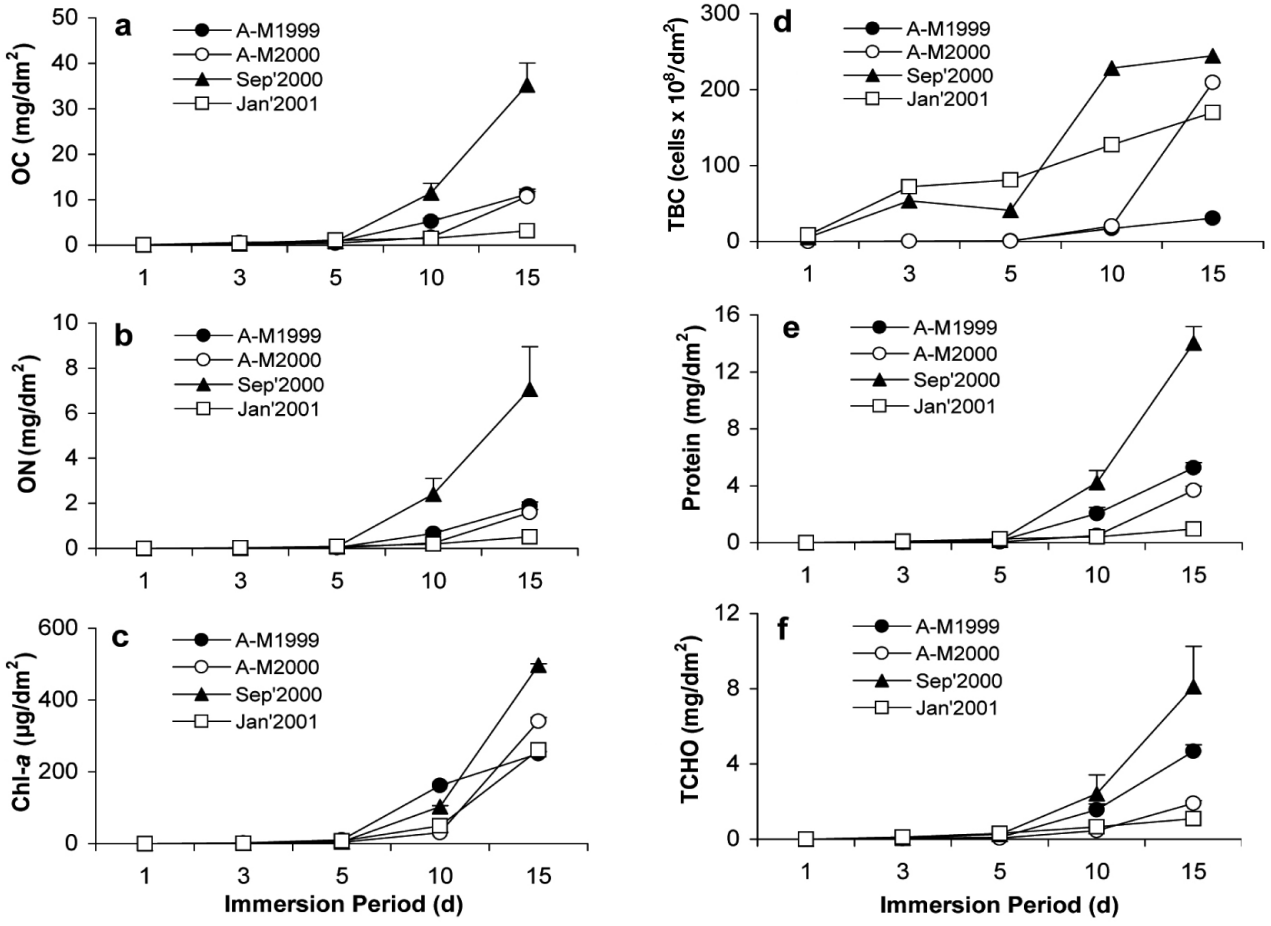

Fig. 1. Changes in (a) biofilm organic carbon (OC), (b) organic nitrogen (ON), (c) chlorophyll *a* (chl *a*), (d) total bacterial counts (TBC), (e) protein and (f) total carbohydrates (TCHO) on stainless steel panels immersed in the surface seawaters of Dona Paula Bay over a period of 15 d in April/May (A–M) 1999, April/May 2000, September 2000 and January 2001. Error bars indicate mean ± SD

chl *a* and TBC. Both the C:N (atomic) and OC:chl *a* (wt:wt) ratios were high at Day 1, then subsequently decreased over the period of immersion (Fig. 2). Concentrations of $TCHO_{SP}$ (Fig. 1f), contribution of $TCHO_{SP}$-C and protein-C to the total OC (Table 2) and the average protein:$TCHO_{SP}$ ratio (Fig. 2) of the biofilm generally increased over the period of immersion.

Aldose concentration and composition

The concentrations of the total aldoses increased over the period of immersion (Fig. 3a). Total aldoses-C accounted for ~10 to 92 and 0.37 to 8.85% of $TCHO_{SP}$-C and OC, respectively (Table 2). The relative distribution (as wt %) of each aldose, however, varied over the period of immersion (Fig. 3b). The wt % contribution of glucose, arabinose and xylose to the total aldoses of the biofilm was high from Days 1 to 5 following immersion. Subsequently, as the period of immersion increased, the contribution of these sugars to the total aldoses decreased, while that of galactose, rhamnose, fucose and ribose generally increased (Fig. 3b).

Table 1. Correlation coefficient (r; n = 20) between various parameters of the biofilm on the stainless steel panels immersed in the surface waters of Dona Paula Bay over a period of 15 d. OC: organic carbon; ON: organic nitrogen; TCHO: total carbohydrates; TBC: total bacterial counts. ***p < 0.001

Parameter	OC	ON	Chl *a*	TCHO	Protein	TBC
OC	1.000	0.996***	0.877***	0.969***	0.997***	0.917***
ON		1.000	0.841***	0.957***	0.992***	0.933***
Chl *a*			1.000	0.877***	0.871***	0.733***
TCHO				1.000	0.982***	0.842***
Protein					1.000	0.906***
TBC						1.000

Table 2. Percent variation in the contribution of aldoses-C, $TCHO_{SP}$-C and protein-C to organic carbon of the biofilm. $TCHO_{SP}$: total carbohydrates by spectrophotometery

Date	Aldoses-C:OC	$TCHO_{SP}$ -C:OC	Protein-C:OC	$TCHO_{SP}$ -C + Protein-C	Aldoses-C:$TCHO_{SP}$-C
April/May 1999					
Day 1	0.68	2.77 ± 0.40	2.82 ± 0.45	5.59 ± 0.23	22.96
Day 3	0.45	4.15 ± 1.14	4.04 ± 1.36	8.19 ± 2.33	10.89
Day 5	1.04	4.36 ± 0.48	8.53 ± 0.27	12.89 ± 0.74	24.55
Day 10	4.06	12.43 ± 1.37	18.31 ± 1.02	30.74 ± 0.51	33.98
Day 15	3.03	16.71 ± 1.28	22.17 ± 2.42	38.88 ± 3.60	18.12
April/May 2000					
Day 1	1.23	4.01 ± 0.86	1.93 ± 0.89	6.13 ± 2.00	29.35
Day 3	0.71	2.31 ± 0.61	4.10 ± 0.85	6.40 ± 1.09	28.14
Day 5	1.96	5.13 ± 1.25	5.82 ± 0.71	10.95 ± 1.89	38.17
Day 10	3.49	10.23 ± 0.23	13.42 ± 0.23	23.65 ± 0.07	33.39
Day 15	3.01	7.20 ± 0.35	16.21 ± 1.26	23.41 ± 1.50	42.09
September 2000					
Day 1	2.19	3.78 ± 1.22	5.19 ± 4.45	8.97 ± 5.67	57.78
Day 3	3.63	6.58 ± 0.90	7.22 ± 0.93	13.81 ± 1.55	57.39
Day 5	8.85	12.00 ± 1.14	10.02 ± 1.13	22.02 ± 2.22	71.19
Day 10	6.31	8.55 ± 0.65	17.23 ± 0.58	25.78 ± 1.23	74.99
Day 15	8.61	10.33 ± 3.89	18.79 ± 1.50	29.12 ± 5.39	92.36
January 2001					
Day 1	0.37	3.27 ± 1.19	2.53 ± 2.17	5.80 ± 3.36	11.46
Day 3	1.76	10.20 ± 5.40	9.69 ± 2.20	19.89 ± 7.60	17.86
Day 5	1.40	11.41 ± 2.47	11.39 ± 4.02	22.80 ± 6.48	12.13
Day 10	2.65	17.67 ± 2.83	12.80 ± 2.62	30.46 ± 5.46	14.97
Day 15	1.98	13.91 ± 2.25	14.31 ± 1.09	28.22 ± 3.34	14.26

Biofilm sources such as bacteria and diatoms were relatively enriched in ribose, fucose and galactose (Table 3). Mangrove leaves showed an abundance of glucose, arabinose and xylose; glucose and mannose were more abundant in macroalgae.

PCA produced 2 factors: Factor 1 accounted for 62.2 to 74.1% of the variance, while Factor 2 gave 11.6 to 19.7% of the variance. The cumulative variance ranged between 80.6 and 85.7%. The plots of PCA

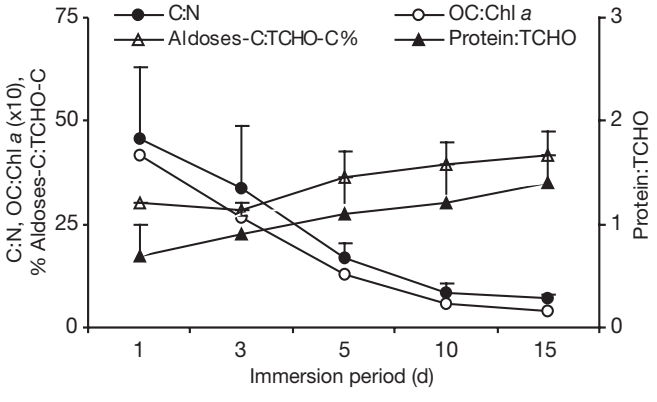

Fig. 2. Average changes in (4 observations) C:N, OC:chl *a* (×10), Protein:$TCHO_{SP}$ and % Aldoses-C:TCHO-C ratios in the biofilm on stainless steel panels. Error bars indicate mean ± SD

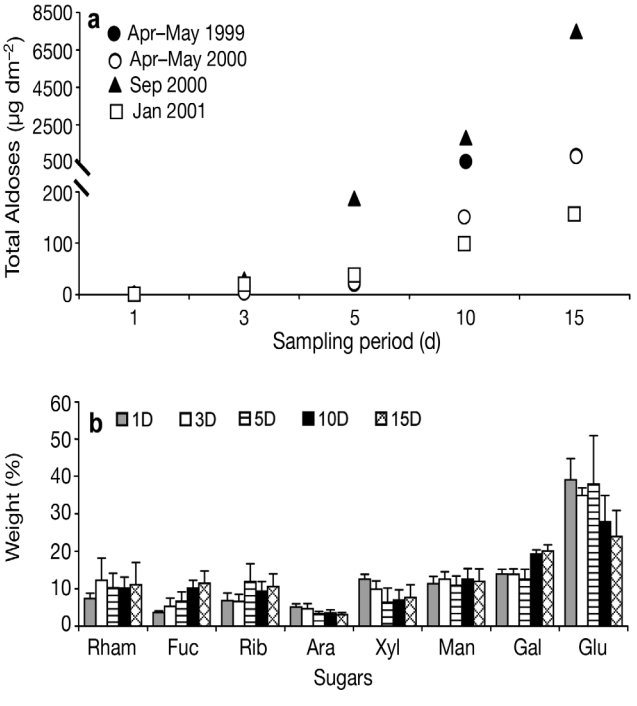

Fig. 3. Changes in average (a) total aldoses and (b) monosaccharide composition of the biofilm developed on stainless steel panels over a 15 d period of immersion in Dona Paula Bay, for all 4 sampling periods. Bars indicate mean ± SD. Rham: rhamnose; Fuc: fucose; Rib: ribose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glu: glucose

loading factors 1 vs. 2 for the biofilm samples clearly separate the variables into clusters, and thus provide better insight into the relationships that exist among the variables (Fig. 4).

DISCUSSION

Biofilm biomass

Our main aim was to characterise carbohydrates during the initial stages of biofilm formation. Therefore, the biofilm development was assessed over a 15 d period of immersion. Normally, long-term immersion periods extending from months to years are generally employed to evaluate macrofouling. Similarly, the removal of biofilm is perhaps the most important step in evaluating biomass on a solid surface immersed in an aqueous environment. Removal of biofilm by brushing is one of the commonly employed techniques (White & Benson 1984, Bhosle et al. 1989, Khandekar & Johns 1990). Studies carried out in our laboratory suggest that scraping using a nylon brush removes ~89 ± 2% of the biofilm developed on metal and non-metal surfaces (Sharma et al. 1990). In our laboratory, a nylon brush was routinely used to remove biofilm from metal and non-metal surfaces (Bhosle et al. 1989, 1993, Bhosle & Wagh 1997, D'Souza & Bhosle 2001, 2003a, b, D'Souza 2004).

Biofilm biomass on SS generally increased over the period of immersion. The ANOVA analysis, however, indicated that the daily ($p > 0.001$) but not the seasonal ($p > 0.01$ to 0.5) variation in biofilm biomass was significant. In contrast, the physical, chemical and biological parameters of the surface seawater did not vary much during any of the sampling periods (D'Souza 2004). The observed increase in biofilm biomass was, therefore, due to an increase in settlement and/or growth of the attached microorganisms. The latter seems likely because the abundance of bacteria (TBC) and microalgae (chl *a*) in the biofilm on SS panels generally increased several-fold over the period of immersion (Fig. 1). This was also supported by the decreasing trends observed for the OC:ON and OC:chl *a* ratios of the biofilm (Fig. 2) (Verity et al. 1992, Oleson & Lundsgaard 1995, Otero et al. 1998, Maksymowska et al. 2000, Verity 2002). Moreover, TBC and chl *a* showed significant relationships with the OC and ON of the biofilm, implying that bacteria and microalgae were important sources for these compounds (Table 1).

Carbohydrate and protein concentrations

Concentrations of both $TCHO_{SP}$ and protein increased over the period of immersion, indicating the production of these compounds by biofilm microorganisms. This was also evident from the significant positive relationships of these compounds with TBC and chl *a.* However, the concentrations of $TCHO_{SP}$ were relatively more abundant in biofilm during the first 5 d period of immersion. This was probably due to the presence of degraded OM, which is generally rich in $TCHO_{SP}$ compared to the protein. Alternatively, the higher amounts of $TCHO_{SP}$ in biofilm during the first 5 d following immersion may be due to induced production of these compounds by the attached microorganisms (Vandevivere & Kirchman 1993).

Microorganisms, especially planktonic diatoms as well as laboratory-grown diatom biofilms, are known to produce large amounts of carbohydrates, particularly during the stationary growth phase and/or under nutrient stress conditions (Myklestad 1977, Bhosle et al. 1993). A number of diatoms including *Navicula*, *Nitzschia*, *Pleurosigm*, *Licmophora* and *Amphora*, etc. were present in the biofilm (D'Souza 2004). Despite the presence of these diatoms carbohydrate production was lower, as is evident from the increasing trends recorded for the protein:carbohydrate ratio of the biofilm over the period of immersion. The relative distribution of carbohydrates and proteins in microorganisms is influenced by their phase of growth. Labora-

Table 3. Monosaccharide composition (wt %) of different biological sources. *Sp. fr.*: *Sphacelaria furicigera*

Sugar	Bacteria		Diatom		Mangrove		Macroalgae	
	Bacteria-1	*Bacteria*-2	*Amphora*	*Navicula*	*Kandelia*	*Rhizophora*	*Gracilaria*	*Sp. fr.*
Rhamnose	7.85	3.34	3.39	4.55	6.10	4.43	1.35	1.40
Fucose	0.00	0.00	11.97	4.27	0.00	0.00	0.00	0.00
Ribose	10.27	31.13	5.45	9.83	0.00	0.00	0.00	0.00
Arabinose	3.87	1.50	0.86	0.27	14.14	9.78	3.60	2.05
Xylose	7.77	1.66	14.67	11.76	16.27	9.27	4.97	4.90
Mannose	29.41	15.66	4.37	10.55	4.50	25.56	6.17	12.05
Galactose	24.49	40.95	24.59	35.02	6.71	9.73	34.69	21.17
Glucose	16.34	5.76	34.69	23.75	52.28	41.22	49.21	58.43

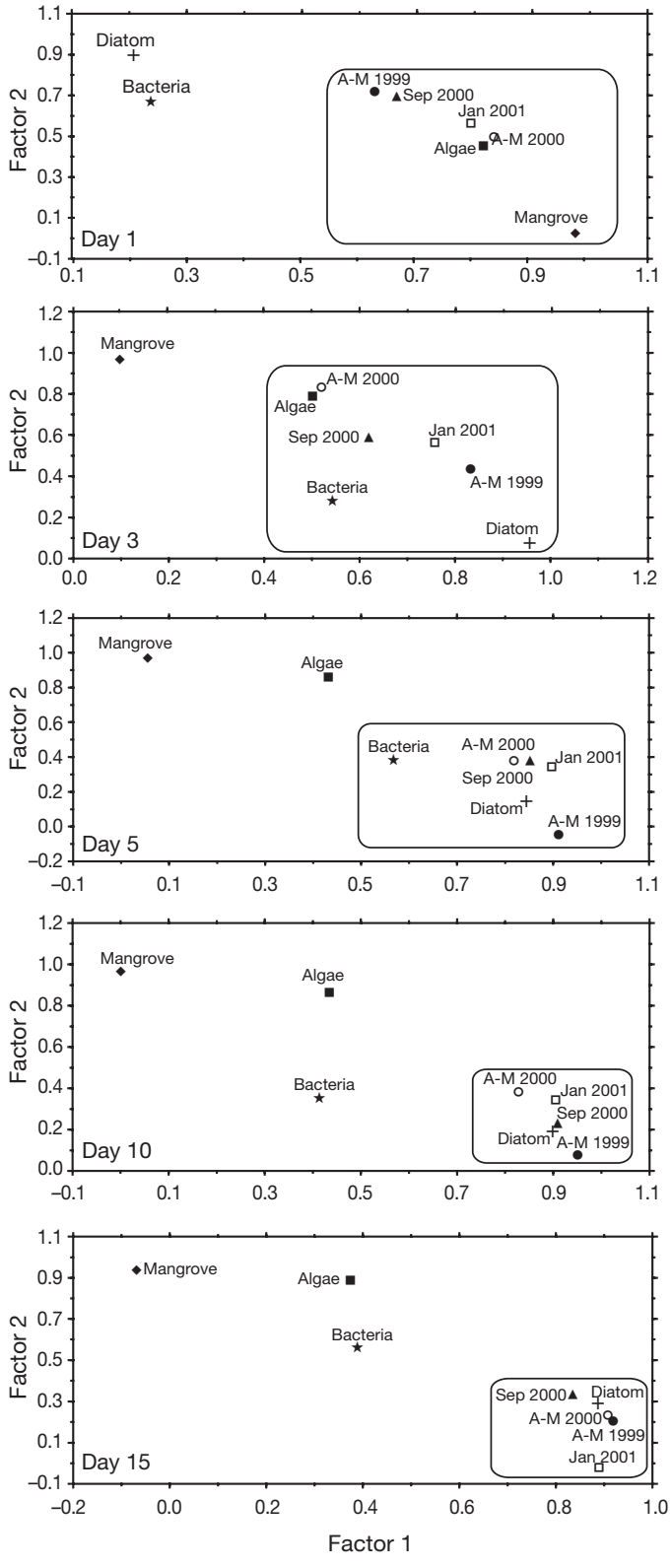

Fig. 4. Plot of loading factors of principal component analysis for the monosaccharide components of the biofilm material developed on stainless steel panels immersed in surface waters of the Dona Paula Bay over a period of 15 d in April/May 1999, April/May 2000, September 2000 and January 2001

tory-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, Rice et al. 2000). Therefore, the observed differences in the relative distribution of $TCHO_{SP}$ and proteins in the biofilm may reflect the effect of the growth phase of the biofilm microorganisms.

Nutrients, especially the levels of nitrate and phosphate in the growth medium, are known to influence the relative distribution of carbohydrates and proteins in laboratory-grown bacteria and microalgae, as well as in natural populations of microorganisms (Corzo et al. 2000). Nitrate and phosphate concentrations in the biofilm were generally low at Day 1 of the immersion period (Rao 2003). Moreover, concentrations of both nitrate and phosphate in the surface seawaters of the study area varied over the period of sampling (D'Souza 2004). Therefore, the observed differences in the levels of $TCHO_{SP}$ and proteins in the biofilm may be related to the nutrient status of biofilm organisms and/or to the nutrient concentrations in surrounding seawaters.

Aldose composition

Neutral sugars were released from their original polymers by hydrolysis. Hydrolysis using HCl or H_2SO_4 is used for the analysis of neutral sugars in natural samples. Both the acids were tested for their ability to release the neutral sugars from biofilm samples (D'Souza 2004). Sugar yields were relatively high when the biofilm samples were subjected to strong hydrolysis using 72% H_2SO_4 at room temperature for 2 h, followed by mild hydrolysis using 1.2 M H_2SO_4 at 100°C for 3 h. This method has also been preferentially employed by others to assess the total hydrolysable sugars in organisms, particles and sediments collected from various aqueous environments (Cowie & Hedges 1984, Tanoue & Handa 1987, D'Souza et al. 2003).

Aldose concentrations increased over the period of immersion, suggesting their production by the fouling microorganisms (Fig. 3a). Nevertheless, the relative contribution of the individual aldoses to the total aldoses varied over the period of immersion (Fig. 3b). This was probably due to changes in the composition of the biofilm organisms, the growth phase, and/or the environmental condition, including the light and nutritional status of the community.

Glucose was generally the most abundant aldose (39%), and its abundance decreased (~39 to ~24 wt %) between Days 5 and 15 of the immersion period (Fig. 3b). This aldose is the major constituent of the

diatom storage polysaccharide β,-1,3 glucan, which is easily degraded by the *in situ* aquatic organisms and/or metabolised during the sinking of diatom cells (Tanoue & Handa 1987). As a result, the observed decrease in glucose may indicate preferential removal of glucose-rich reserve polymers by heterotrophic microorganisms and/or its uptake by the respiring diatoms (Tanoue & Handa 1987). Further, grazing can also reduce the glucose content. For example, Cowie & Hedges (1996) reported selective utilisation of glucose-rich polymers by copepod zooplankton when they are fed with the diatom *Thalassiosira weissflogii*. Moreover, D'Souza & Bhosle (2001) observed that the glucose content in natural populations of diatoms decreased with the increase in nitrate and phosphate concentrations. Therefore, it is possible that the decrease in the glucose content of the biofilm was related to the nutrient status of the biofilm organisms.

Arabinose and xylose were relatively more abundant (~19 wt %) in biofilm at Day 1 of the immersion period (Fig. 3b). These sugars do not contribute significantly to either the cell or cell wall material of diatoms, but are abundant in terrestrial plants as constituents of arabinoxylan, arabino glucuronoxylan, etc. (Cowie & Hedges 1984, Ogier et al. 2001). Moreover, these aldoses were relatively enriched in mangrove leaves, especially *Kandelia* (Table 3). The relative abundance of these sugars, therefore, may suggest the presence of terrestrial material in the biofilm developed on the SS at Day 1 of the immersion period (Hedges et al. 1985, Ogier et al. 2001).

Decrease in glucose, arabinose and xylose was associated with a relative increase in fucose, rhamnose, galactose and ribose (Fig. 3b). In some of the diatoms, glucose-rich polymers are replaced by fucose-rich storage polysaccharides (Percival 1970). Moreover, fucose is one of the abundant aldoses among the cell wall polysaccharides of the diatoms *Nitzschia* sp. and *Navicula* sp. (Hecky et al. 1973). The abundance of both these diatoms in the biofilm on the SS panels increased over the period of immersion (D'Souza 2004). Therefore, the increase in fucose content in the biofilm over the period of immersion probably indicates the influence of these diatoms.

The abundance of rhamnose (5.8 to 19.9%), galactose (~24 to 41%) and ribose (7.4 to 19.1%) in the biofilm also increased over the period of immersion. These sugars are relatively abundant in microorganisms, especially bacteria. For example, *Pseudomonas* sp., *Azotobacter* sp. and *Caulobacter* sp. are enriched in rhamnose (24 to 44%) (Hicks et al. 1994). Similarly, ribose (11 to 61%) and galactose (~24 to 41%) are enriched in some unidentified marine bacteria, including fouling bacteria (10 to 30%) (Cowie & Hedges 1984, D'Souza 2004). These bacteria were found in the biofilm developed on metal panels immersed in the surface waters of the study area (D'Souza & Bhosle 2003a). Therefore, the observed increase in the contribution of rhamnose, fucose, ribose and galactose to the total aldoses over the period of immersion indicates the contribution from bacteria and diatoms.

In order to further define the major sources of biofilm OM, the aldose composition data of the biofilm and the source samples were further processed using PCA. Plots of the PCA loadings for Factor 1 vs. Factor 2 separated the variables into clusters (Fig. 4). The biofilm samples merged with mangroves and macroalgae, indicating the contribution of mangroves and macroalgae to the biofilm OM that developed on the SS plates at Day 1 of the immersion period (Fig. 4). Subsequently, at Day 3 of immersion, the biofilm samples were roughly distributed inside the square along with diatoms, bacteria and macroalgae, indicating that the aldoses were mostly derived from these sources. At Day 5, further change in the biofilm community structure was revealed by the close clustering of the biofilm samples with diatoms and bacteria. As the immersion period continued (10 and 15 d), diatoms formed a close cluster with biofilm samples, thereby indicating the predominance of OM derived from microalgae.

Nature of the biofilm organic matter

The contribution of $TCHO_{sp}$-C, protein-C and total aldoses-C to the total OC in a sample is a useful tool to evaluate the degradation state of the OM (Cowie et al. 1995, Skoog & Benner 1997, D'Souza & Bhosle 2003b). Carbohydrates and proteins account for 30 to 70% of the OM in all types of fresh marine and terrestrial sources; these values decrease during the degradation of OM (Hedges et al. 1994, Cowie et al. 1995, Pantoja & Lee 1999). At Day 1 following immersion, the contribution of $TCHO_{SP}$-C plus protein-C and the total aldoses-C to the biofilm OC varied between ~5.6 and 8.9 and 0.37 and 2.2%, respectively (Table 2). Compared to the live marine and terrestrial materials, these values were low, suggesting the presence of extensively degraded OM in the biofilm developed on SS at Day 1 following immersion. As the immersion period continued, the contribution of $TCHO_{SP}$-C plus protein-C (~23 to 39%), and total aldoses-C (~2 to 9%) to the total biofilm OC increased, implying a relative abundance of fresh biogenic OM in the biofilm (Table 2). Nevertheless, only ~6 to 39% of OC was accounted for by carbohydrates and proteins in the biofilm samples, which means that ~61 to 94% of the biofilm OC remained uncharacterised. Although lipids may account for a certain portion, much of the OM remained unidentified. However, this was not an

unusual observation. For example, Wang et al. (2004) reported that 35 to 49% of the OC of estuarine samples remained uncharacterised. Similarly, in Delaware Bay, 61 to 79% of the OC remained uncharacterised (Harvey & Manino 2001). This molecularly uncharacterised OM may indicate the presence of biologically and chemically modified OM and/or failure of the modern hydrolysis methods (Skoog & Benner 1997, Ogawa et al. 2001). It will be interesting to determine the role of this OM in the development of biofilm.

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