

## **Chemical characterization of the marine conditioning film**

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### **Abstract**

Conditioning film developed on the glass panels was analyzed for organic carbon and nitrogen, total carbohydrates, aldoses, uronic acids and proteins. These compounds showed non-linear increase over the period of immersion. Total carbohydrates-C, aldoses-C, uronic acid-C, and protein-C accounted for 5.59 to 23.50 %, 0.6 to 5 %, 1 to 5 %, and 4 to 14 % of organic carbon, respectively, while aldoses-C accounted for 10 to 72 % of the total carbohydrate-C. Aldoses such as rhamnose, arabinose, xylose, mannose and glucose were generally present in the conditioning film. A principal component analysis based on wt % aldoses established three factors that contributed ~77 % of the total variance. The first factor was influenced by the abundance of rhamnose and accounted for 41 % of the total variance. The second factor was influenced by the concentrations of arabinose and xylose, whereas the third factor was controlled by ribose and galactose thereby implicating the role of terrestrial and microbial input to the conditioning film. These changes in the chemistry of the conditioning film may play some role in influencing microbial adhesion to surfaces.

**Key words:** Conditioning film, glass surface, carbohydrates, aldoses, uronic acids

## 1. Introduction

Surfaces immersed in aquatic environment adsorb dissolved organic matter (Baier, 1972; Loeb and Neihof, 1975; Taylor et al., 1997; Bhosle et al., 2005). This is defined as the conditioning film or the molecular film (Baier, 1972; Loeb and Neihof, 1975; Taylor et al., 1997). Conditioning film is composed of many compounds including glycoproteins (Baier, 1980), humic material (Loeb and Neihof, 1975), proteins, lipids, nucleic acids, polysaccharides and aromatic amino acids (Taylor et al., 1997) and/or unspecified macromolecules (Zaidi et al., 1984). Of these constituents, proteins were the first compounds followed by carbohydrates to adsorb onto the stainless steel panels immersed in seawater (Compere et al., 2001, Poleunis et al., 2002, 2003). It appears that cleaning procedures and substratum surface properties may influence the chemical composition and the amount of the adsorbed material (Little and Zsolnay, 1985; Taylor et al., 1997; Compere et al., 2001). Changes in the chemical composition of the conditioning film may influence surface charge, wettability, surface free energy and surface roughness of the substratum. These changes may play an important role in subsequent colonization by microorganisms, especially bacteria (Dexter, 1979; Baier, 1980; Fletcher and Marshall, 1982; Schneider, 1997, Bakker et al., 2004).

Carbohydrates are common and important structural and storage constituents in marine and terrestrial organisms. They account for ~ 10 to 50 % of dissolved organic carbon in marine waters (Pakulski and Benner, 1994; Amon and Benner, 2003). Storage carbohydrates are rapidly utilized by the in situ organisms (Handa and Tominaga, 1969; Tanoue and Handa, 1987). This results in the accumulation of relatively less degradable structural carbohydrates in marine waters. Abundance and composition of neutral sugars and its composition are useful tools to assess the sources of organic matter (Cowie and Hedges, 1984; Skoog and Benner, 1997; D'Souza et al., 2005).

Carbohydrate polymers appear to play an important role in biofilm development. They are one of the most abundant constituents of the conditioning film or molecular film (Compere et al., 2001). Carbohydrate polymers adsorbed to surfaces may also contain uronic acid, pyruvate, sulphate and proteins (Decho, 1990). It is well known that these compounds may play an important role in the subsequent colonization by microorganisms. However, earlier studies have mostly focused their attention on the characterization of the bulk parameters such as carbon and nitrogen and/or the presence of the specific functional groups in the conditioning film using surface analytical techniques. These studies have

provided useful information on the bulk chemical composition of the conditioning film. Nevertheless, little information is available on the molecular composition, nature and short-term temporal variability of the organic matter in general, and carbohydrates and non-carbohydrate constituents in particular, of the conditioning film (Bhosle et al., 2005). A proper understanding of the chemical composition and kinetics of the conditioning film formation is required to better explain the biofilm growth and microbial adhesion mechanisms. Furthermore, the amount and relative contribution of individual organic constituents associated with organic carbon such as amino acids and sugars and their temporal variations provide information on the nature and degradation state of the organic matter (Cowie et al., 1995; Bhosle et al., 2005).

The aims of the present research were 1) to study the dynamics of carbohydrates, uronic acid and proteins in the adsorbed organic matter, 2) to determine the changes in the composition of neutral carbohydrates over the period of immersion and 3) to use the concentration and composition of carbohydrates to assess the nature of the adsorbed organic matter. To the best of our knowledge, this study presents the first data set on the dynamics of total carbohydrate, aldoses and uronic acid in the conditioning film.

## **2. Materials and Methods**

### *2.1. Chemicals and reagents*

Glass cover slips were purchased from Blue Star, India. Standard sugars and, myo-inositol were obtained from Sigma (St. Louis, MO, USA). Sodium carbonate, NaOH, HCl and methanol were of high purity AR grades and were purchased from s.d. Fine Chemicals, Mumbai. Methanol was double distilled before use. Distilled water was purified using UV-Milli-Q water purification system (Millipore, Bangalore, India).

### *2.2. Preparation and deployment of the glass cover slips*

The development of the conditioning film was studied using glass cover slips (hereafter called as glass panels). Before use the glass panels (22 X 60 mm) were thoroughly cleaned with chromic acid solution for 5 hours, washed with UV treated Milli-Q purified distilled water followed by methanol and dried in an oven. Individual panels were covered with pre-ashed aluminum foil (450 °C for 5 h). The panels were then kept in a muffle furnace and ashed at 450 °C for 5 hours.

### *2.3. Deployment of the glass panels in running seawater*

Glass panels were fixed onto a clean PVC racks (10.6 X 40 X 4 cm). Each rack had 28 slots to hold 56 glass panels. Before immersion, PVC racks were covered with PVC lids in order to avoid air – water interface (Fig. 1). The entire assembly was then immersed in a glass tank (length 90.15 cm, breadth 45.75 cm, height 45.75 cm) filled with seawater. The glass tank was connected to a reservoir tank in which the surface seawater of Dona Paula Bay, West Coast of India, was pumped. Seawater flow in the glass tank was set at 500 ml/min. Once the PVC racks containing glass panels were properly installed in the glass tank, the PVC cover was removed. The panels were deployed during July and November 2006.

### *2.4. Sampling*

Panels were retrieved at 0, 2, 4, 6, and 8 h following immersion. Panels were dipped and immediately removed and defined as 0 h. During each sampling 80 glass panels were retrieved from the PVC rack using clean forceps and transferred to clean beakers kept in the tank. The beakers with the panels were removed from the tank. The panels were then rinsed thoroughly by passing through a series of 4 beakers (100 ml) each containing 100 ml of the UV treated Milli-Q purified water so as to remove the loosely attached organic matter. The panels were then transferred to clean glass test tubes, covered with aluminum foil and stored at – 20 °C until analysis. A set of unused panels were used as blanks for all the parameters analyzed.

### *2.5. Bulk parameters analysis*

Organic carbon and organic nitrogen of the conditioning film were estimated following the methods of Parsons et al., (1984) and Smart et al., (1983), respectively. Total carbohydrate was estimated following the phenol-sulphuric acid method (Dubois et al., 1956). Uronic acids were estimated by the method of Filisetti-Cozzi and Carpita (1991). Protein content was analyzed using the method of Smith et al., (1985). The coefficient of variations of these methods based on replicate (5) analysis was better than 10 %.

### *2.6. Aldose composition*

Glass panel with the adsorbed organic matter was crushed into pieces, using a clean glass rod and transferred into a glass ampoule containing 2 ml of 2 N HCl (Bhosle et al.,

1995). The ampoule was flushed with nitrogen and sealed. Sample was then hydrolyzed for 3 h at 100 °C. Liberated sugars were then converted to alditol acetates and analysed using a capillary gas chromatograph (HP GC Model 6890) as described earlier (D'Souza and Bhosle, 2003; D'Souza et al., 2005). In this study the total aldoses has been defined as the sum of all the identified monosaccharides. The contribution of individual aldose to the total aldoses was expressed as wt %. Analytical variability of the GC method was greater than  $\pm 6$  %, whereas it was higher than  $\pm 10$  % for the GC plus sample preparation method.

### *2.7. Statistical method*

A simple regression analysis was carried out to assess the relationships between various parameters (Sokal and Rohlf, 1981) using the Excel software program loaded on the personal computer. Analysis of variance (ANOVA) was carried out to understand the temporal and seasonal variations in the measured parameters. A principal component analysis (PCA) was performed on a personal computer using the statistical software package version 5.0 (Statsoft, Inc.). The PCA was carried out using the wt % composition of the aldoses (D'Souza et al., 2005).

## **3. Results and discussion**

### *3.1. Organic carbon and nitrogen in the conditioning film*

Concentration of organic carbon and nitrogen increased for the first 4 h and then decreased for the remaining period of immersion during both the July and November, 2004 sampling (Fig. 2a & b). However, the observed increase in organic carbon was not significant for time ( $F=1.87$ ;  $P=0.20$ ) and the seasons ( $F=2.14$ ;  $P=0.17$ ), whereas nitrogen showed significant temporal variations ( $F=3.39$ ;  $P=0.06$ ). The concentration of organic carbon was generally greater than that recorded for organic nitrogen. This compares well with the earlier studies reporting the relative abundance of carbon compared to organic nitrogen. For example, Compere et al., (2001) observed high abundance of organic carbon compared to organic nitrogen in the conditioning film developed on stainless steel. Similarly, Bhosle et al., (2005) reported relatively higher concentrations of organic carbon compared to organic nitrogen in the conditioning film developed on glass panels deployed in surface seawaters of the Dona Paula Bay, west coast of India. C/N ratio of the conditioning film was generally high and varied from 4 to 16. Higher abundance of organic carbon relative to organic nitrogen results in higher C/N (atomic) ratio thereby indicating the presence of

degraded organic matter and/or terrestrial organic matter (D'Souza and Bhosle, 2001; Bhosle et al., 2005). Scanning electron microscopic observations of the conditioning film did not reveal the presence of identifiable microorganisms thereby suggesting that the organic constituents of the conditioning film were not influenced by the presence of microorganisms.

### 3.2. *Total carbohydrates and proteins*

Carbohydrates of the conditioning film showed increasing trends over the period of immersion (Fig. 2c). Proteins of the conditioning film increased for the first 6 hours and then showed some decrease over the remaining period of immersion. Concentrations of carbohydrates and proteins were relatively higher in July, compared to November. This was probably due to higher primary production in July as compared to November (Padmavati and Goswami 1996). Temporal (hourly) variations in carbohydrates were not significant but seasonal variations were significant ( $F=11.03$ ;  $p=0.005$ ) probably due to differences in primary production. Carbohydrate-carbon accounted for 5 to 23 % of the organic carbon and the contribution of carbohydrates to the organic carbon was relatively more in July, (Table 1). Carbohydrates and proteins showed good correlation with organic carbon ( $R^2 = 0.449$ ;  $p=0.01$ ;  $R^2= 0.448$ ;  $p= 0.01$ ) (Table 2). Protein-carbon accounted for 4 to 14 % of the organic carbon (Table 1). Proteins compared to carbohydrates are more abundant in the conditioning film during the first few hours following deployment (Baier, 1980, Compere et al., 2001). In the present study we observed considerable variations in the relative abundance of these major biochemical components in the conditioning film that developed on glass (Table 1). This indicates that the relative abundance of these compounds can vary during the initial stages of biofilm formation.

### 3.3. *Uronic acids*

Uronic acids are carboxylated forms of sugars on a polysaccharide. The uronic acids confer an overall negative charge and acidic properties to the polysaccharides. Many microorganisms and terrestrial plants produce uronic acids. (Decho, 1990; Hung et al., 2003). Concentration of uronic acids in the conditioning film increased over the period of immersion and values were relatively high in July as compared to November (Fig. 2e). This probably suggests increasing adsorption of uronic acids, as they are highly surface-active compounds. Acidic polysaccharides such as uronic acids may play an important role in regulating the supply of nutrients and organic carbon in biofilm microorganisms (Decho, 1990). Uronic acids are also known to influence the adhesion of bacteria (Tsuneda et al.,

2003). Uronic acids accounted for 1 to 5 % of organic carbon of the conditioning film. Uronic acids gave fairly good relationships with organic carbon ( $R^2=0.465$ ;  $p=0.01$ ) and total carbohydrates ( $R^2=0.868$ ;  $p=0.001$ ) (Table 2) indicating that these compounds were common constituents of organic carbon (Table 1).

### 3.4. Total aldoses

Concentration of aldoses in the conditioning film generally increased over the period of immersion during July, whereas in November, aldose concentration increased for the first 4 h followed by a small decrease at 6 and 8 h following immersion (Fig. 2 f). Aldoses showed a fairly good relation with total carbohydrates ( $R^2=0.463$ ;  $p=0.001$ ), (Table 2). Aldoses-C accounted for 10 to 72 % of total carbohydrate and 0.6 to 5.6 % of organic carbon (Table 1). Aldoses such as arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose and xylose, were detected in the conditioning film (Fig. 3).

These sugars are commonly found in dissolved and particulate organic matter and in terrestrial and marine organisms (Cowie and Hedges, 1984; Mopper et al., 1995; Borch and Kirchman, 1997; D,Souza and Bhosle, 2001). The contribution of individual sugar to total aldoses, however varied during the duration of immersion and the season of the immersion. Rhamnose was most abundant in the conditioning film. Rhamnose is more abundant in dissolved organic matter of estuaries and rivers. In contrast, the fucose is more abundant in the marine dissolved organic matter (Benner and Opsahl, 2001). The relative abundance of rhamnose probably suggests the influence of riverine dissolved organic matter (Fig. 3) (Skoog and Benner, 1997). This seems possible as the sampling location is influenced by run-off from the Mandovi River. Furthermore, rhamnose is also recognized as a surface active carbohydrate (Mopper et al., 1995). It appears that phytoplankton exudates are relatively enriched in surface active compounds like rhamnose (Mopper et al., 1995; Skoog and Benner, 1997).

Aldose yield (yield = Total aldoses-C/organic carbon x 100) is a useful tool to assess the reactivity of organic matter (Skoog and Benner, 1997; Amon and Benner, 2003). Aldose yields for freshly derived organic matter is ~ 40% (Skoog and Benner, 1997). Low aldose yields are generally recorded for the degraded organic matter. Most of the values of the total aldoses-C % observed in the conditioning film are relatively low suggesting that the conditioning film was mostly dominated by degraded organic matter (Table 1).

In order to further differentiate the aldoses, data were processed using principal component analysis (PCA). Three factors were obtained which together explained 76 % of the cumulative variance (Table 3). The first factor was mostly influenced by the changes in rhamnose as evident from high positive factor loading obtained for this sugar. As described above this monosaccharide is a surface active agent, and is also abundant in the degraded OM. This again clearly demonstrates the importance of surface active carbohydrates and degraded organic matter in the conditioning film. The second factor showed high positive factor loadings for arabinose and xylose. These two sugars are important constituents of carbohydrates of terrestrial material. This suggests that the second factor was controlled by the terrestrial input in the conditioning film. On the other hand, the third factor showed high negative factor loadings for ribose and galactose. These two sugars are relatively more abundant in organic matter derived from bacteria and phytoplankton. Therefore the third factor was influenced by the organic matter derived from bacteria and phytoplankton.

#### **4. Conclusions**

Conditioning film formed on a hydrophilic glass panels was chemically characterized. Carbohydrates, uronic acids and proteins of the conditioning film varied temporally and seasonally. Aldose concentrations, composition and yields indicate that the conditioning film was derived from degraded dissolved organic matter. These changes in the chemical composition of the conditioning film, on the other hand, may influence surface charge, wettability and surface free energy of the substratum surface. These changes are then likely to play an important role in the subsequent colonization by bacteria.

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Table 1

Variation in the contribution of TCHO-C, aldoses-C, uronic acid (URA)-C, protein-C to organic carbon, aldoses-C to total carbohydrate carbon and TCHO/protein ratio

Sampling months	Sampling time	TCHO-C/OC %	Aldose-C/OC %	URA-C/OC %	Protein-C/OC %	Aldose-C/TCHO-C %	TCHO/Protein
July	0	13.36 ± 0.46	1.85 ± 0.10	4.01 ± 0.03	8.84 ± 0.41	18.92 ± 1.61	2.76 ± 0.35
	2	13.45 ± 0.29	4.72 ± 0.53	4.16 ± 0.21	13.96 ± 0.56	50.27 ± 2.15	3.24 ± 0.18
	4	13.19 ± 0.58	2.42 ± 0.84	4.72 ± 0.28	12.34 ± 0.24	23.30 ± 1.03	4.05 ± 0.16
	6	14.43 ± 0.79	4.16 ± 0.57	4.47 ± 0.17	11.07 ± 0.89	34.90 ± 2.81	3.29 ± 0.21
	8	15.20 ± 0.52	5.59 ± 0.45	5.07 ± 0.27	11.30 ± 0.69	62.11 ± 2.39	2.59 ± 0.14
November	0	12.36 ± 0.53	5.32 ± 0.23	1.63 ± 0.12	5.44 ± 0.23	72.52 ± 4.68	2.73 ± 0.23
	2	13.88 ± 0.50	3.09 ± 0.26	1.62 ± 0.09	3.45 ± 0.50	38.56 ± 1.16	2.89 ± 0.21
	4	5.59 ± 0.22	1.22 ± 0.20	1.39 ± 0.06	4.41 ± 0.25	22.00 ± 1.30	3.15 ± 0.26
	6	10.14 ± 0.41	0.65 ± 0.06	1.43 ± 0.10	8.23 ± 0.69	10.44 ± 0.68	4.25 ± 0.22
	8	23.50 ± 0.70	3.48 ± 0.23	1.94 ± 0.09	3.91 ± 0.07	45.10 ± 2.07	2.85 ± 0.10

Table 2  
 Correlation coefficient (  $R^2$  ) for various parameters of conditioning film  
 developed over a period of 8 h on glass surface (n = 10)

Parameters	Carbon	Carbohydrates	Uronic acid	Protein	Aldoses
Carbon	1.000	0.449 <sup>b</sup>	0.465 <sup>b</sup>	0.408	0.295
Carbohydrate		1.000	0.868 <sup>a</sup>	0.879 <sup>a</sup>	0.463 <sup>b</sup>
Uronic acid			1.000	0.855 <sup>a</sup>	0.439 <sup>b</sup>
Protein				1.000	0.612 <sup>a</sup>
Aldoses					1.000

<sup>a</sup>  $p \leq 0.001$ , <sup>b</sup>  $p \leq 0.01$

Table 3  
 Factor loading of varimax-rotated factors extracted by PCA, and  
 their eigenvalues and variance

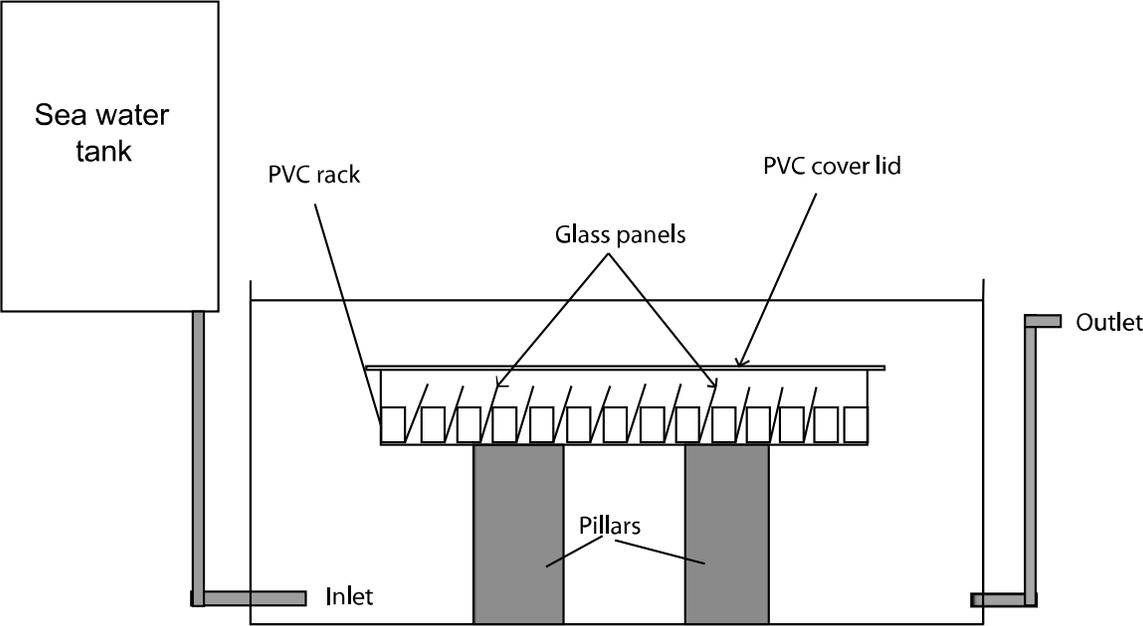
Factor	1	2	3
Rhamnose	0.7172	-0.2901	0.4236
Fucose	0.0680	0.4264	0.3357
Ribose	0.2314	-0.0185	-0.8454
Arabinose	-0.0604	0.8089	-0.2396
Xylose	-0.4373	0.7141	0.2592
Mannose	0.0297	-0.9442	0.0700
Galactose	0.0387	0.0911	-0.8964
Glucose	-0.5701	0.4508	0.4940
Eigenvalue	4.9422	2.3421	1.9230
Variance (%)	41.1848	19.5174	16.0250
Cumulative variance (%)	41.1848	60.7022	76.7272

### **Caption to figure**

**Fig. 1.** Schematic diagram of the experimental setup for conditioning film development in running sea water system.

**Fig. 2.** Variations in organic carbon (OC) (a), organic nitrogen (ON) (b), total carbohydrate (TCHO) (c), protein (d), uronic acid (URA) (e), and aldoses (f) of the conditioning film developed on glass panels immersed over a period of 8 h in the surface seawater of Dona Paula Bay during July and November 2006. Bar indicates mean  $\pm$  standard deviation.

**Fig. 3.** Average changes in monosaccharide composition of the conditioning film developed on glass panels immersed over a period of 8 h in the surface seawater of Dona Paula Bay. Rha = rhamnose; Fuc = fucose; Rib = ribose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glu = glucose. Bar indicates mean  $\pm$  standard deviation.



**Fig.1.**

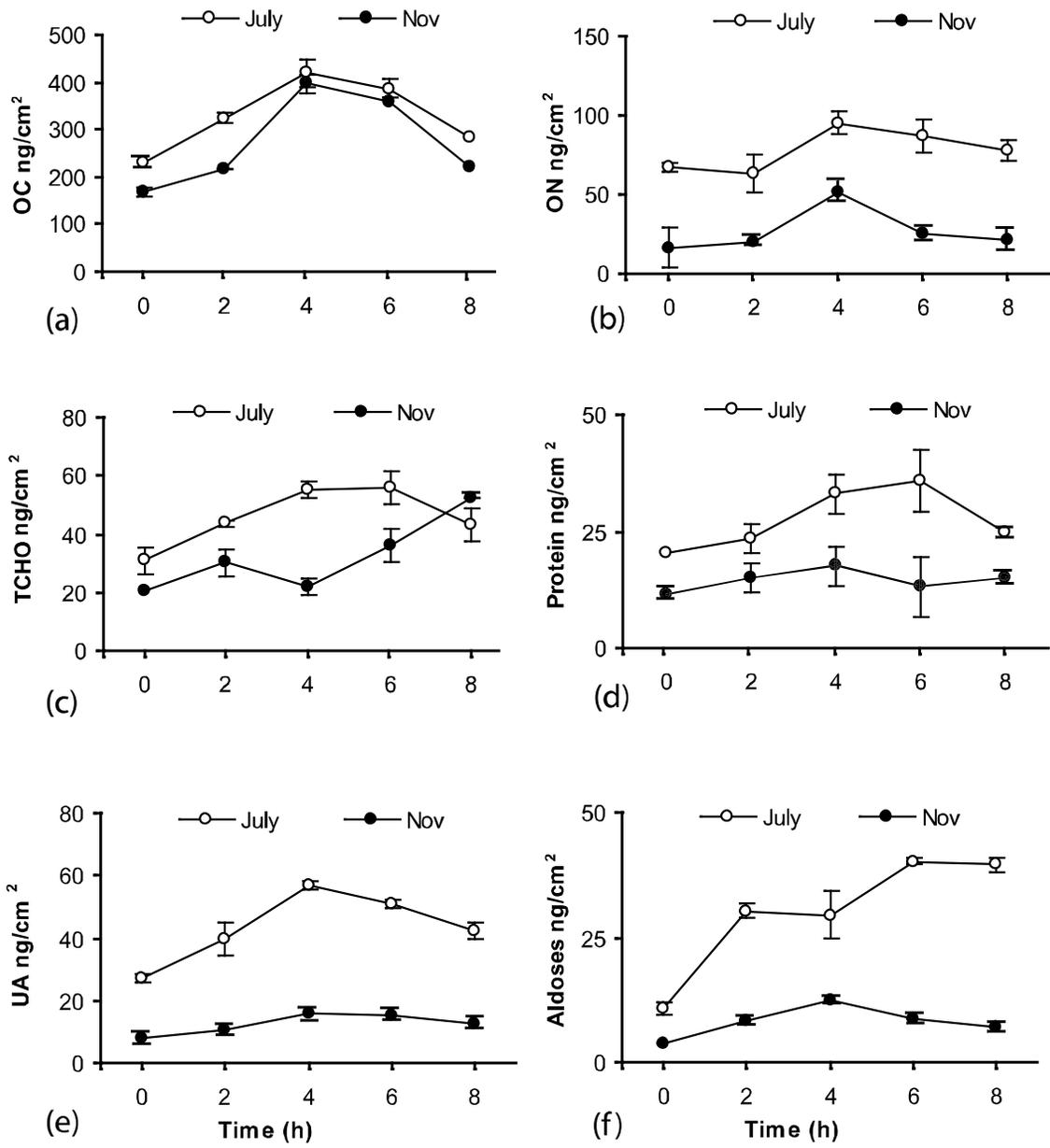
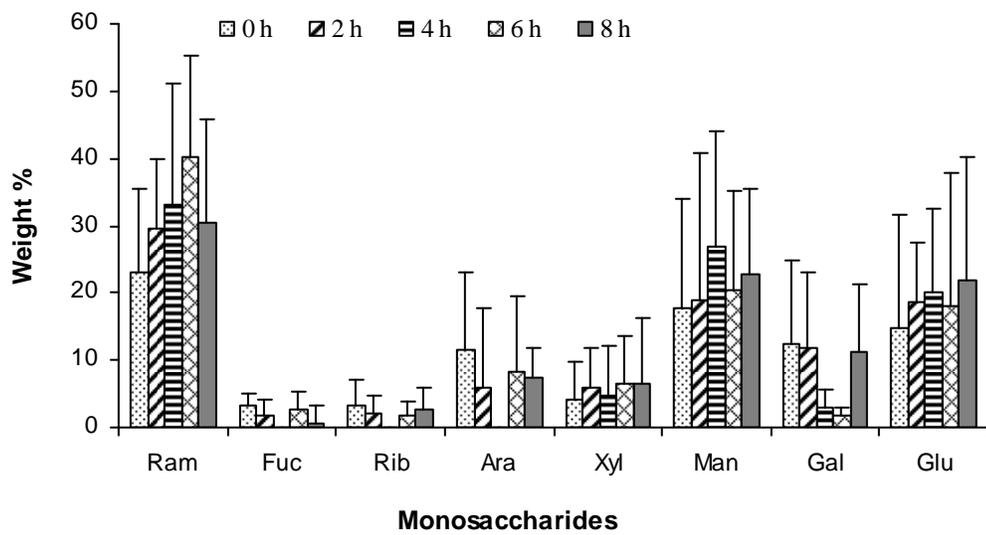


Fig. 2.



**Fig. 3.**