PRODUCTION AND FATE OF CARBOHYDRATE IN AQUATIC ENVIRONMENTS

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IN

MICROBIOLOGY

BY

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UNDER THE GUIDANCE

OF

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CERTIFICATE

This is to certify that the thesis entitled "**Production and fate of carbohydrate in aquatic environments**" submitted by **Mr. Vishwas B. Khodse** for the award of the degree of Doctor of Philosophy in Microbiology is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.

Place: Dona Paula
Date: December 10, 2010

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No Corrections have been suggested by the referees

2/12/2011
External Examiner

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STATEMENT

As required under the university ordinance 0.19.8 (vi), I state the present thesis entitled "**Production and fate of carbohydrate in aquatic environments**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

VBKhodse

(Vishwas B. Khodse)

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(Vishwas B. Khodse)

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List of abbreviations used

CO_2	Carbon dioxide
DOM	Dissolved organic matter
POM	Particulate organic matter
OM	Organic matter
DOC	Dissolved organic carbon
POC	Particulate organic carbon
TPN	Total particulate nitrogen
PSA	Phenol sulphuric acid
MBTH	3-methyl-2 benzo thiozoline hydrazone
TPTZ	2,4,6-tripyridyl-s-triazine
GC	Gas chromatography
HPLC	High performance liquid chromatography
PAD	Pulsed amperometric detection
LMW	Low molecular weight
HMW	High molecular weight
EPS	Exopolysaccharide
BSS	Basal salt solution
OD	Optical density
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
dNTP	Deoxyribonucleotide triphosphate
MCHO	Free monosaccharides
TDCHO	Total dissolved carbohydrate
HCl-TDCHO	HCl-hydrolyzable total dissolved carbohydrate
DPCHO	Dissolved polysaccharides
HCl-DPCHO	HCl-hydrolyzable dissolved polysaccharides
HR-DPCHO	HCl-resistant dissolved polysaccharides
DURA	Dissolved uronic acids

TPCHO	Total particulate carbohydrate
TNPCHO	Total neutral particulate carbohydrate
TPURA	Total particulate uronic acids
Acid-TPCHO	Dilute acid soluble particulate carbohydrate
Alkali-TPCHO	Dilute alkali soluble particulate carbohydrate
Res-TPCHO	Residual particulate carbohydrate
Rham	Rhamnose
Fuc	Fucose
Rib	Ribose
Arb	Arabinose
Xyl	Xylose
Man	Mannose
Gal	Galactose
Glu	Glucose
GalN	Galactosamine
GlcN	Glucosamine
ManN	Mannosamine
Mur	Muramic acids
Chl *a*	Chlorophyll *a*
PP	Particulate protein
TPAS	Total particulate amino sugars
DW	Dry weight
SPM	Suspended particulate matter
TBC	Total bacterial count/ abundance
BP	Bacterial production
BB	Bacterial biomass
DO	Dissolved oxygen
BR	Bacterial respiration
BGE	Bacterial growth efficiency
EDTA	Ethylenediamine tetraacetic acid
PCA	Principal component analysis

Dedicated to my parents

1. Introduction

1.1 Marine carbon cycle

Two-thirds of the earth's surface is covered by water, and approximately half of the global primary production is produced in the marine ecosystem.

The most important process responsible for primary production is oxygenic photosynthesis by phytoplankton (Hedges, 1992). Phytoplankton are microscopic plants, mostly algae, which live suspended in the water column. All plants use light, to convert carbon dioxide (CO_2) and water into organic compounds and oxygen during the photosynthesis process. This is defined as organic matter production. Carbohydrates are among the first organic compounds formed during the photosynthesis. Carbohydrates serves as major source of energy for heterotrophic organisms, and provide 60% of their energy requirements. Carbohydrates are involved in structure, energy and growth regulating processes of the living organisms. The organic carbon produced in the aquatic environment is respired in the marine food web (Fig. 1.1).

In aquatic environments, dissolved organic matter/carbon (DOM or DOC) and particulate organic matter/carbon (POM or POC) are mostly derived from various sources including algal extracellular secretions, viral infection and lysis of bacteria and phytoplankton cells, sloppy zooplankton feeding, microbial degradation of organic matter, river run off and physical transport of terrestrial organic matter (Guo and Santschi, 1997; Opsahl and Benner, 1998). In the aquatic food chain carbon and energy is channelled through algae, to herbivores and on to higher trophic levels.

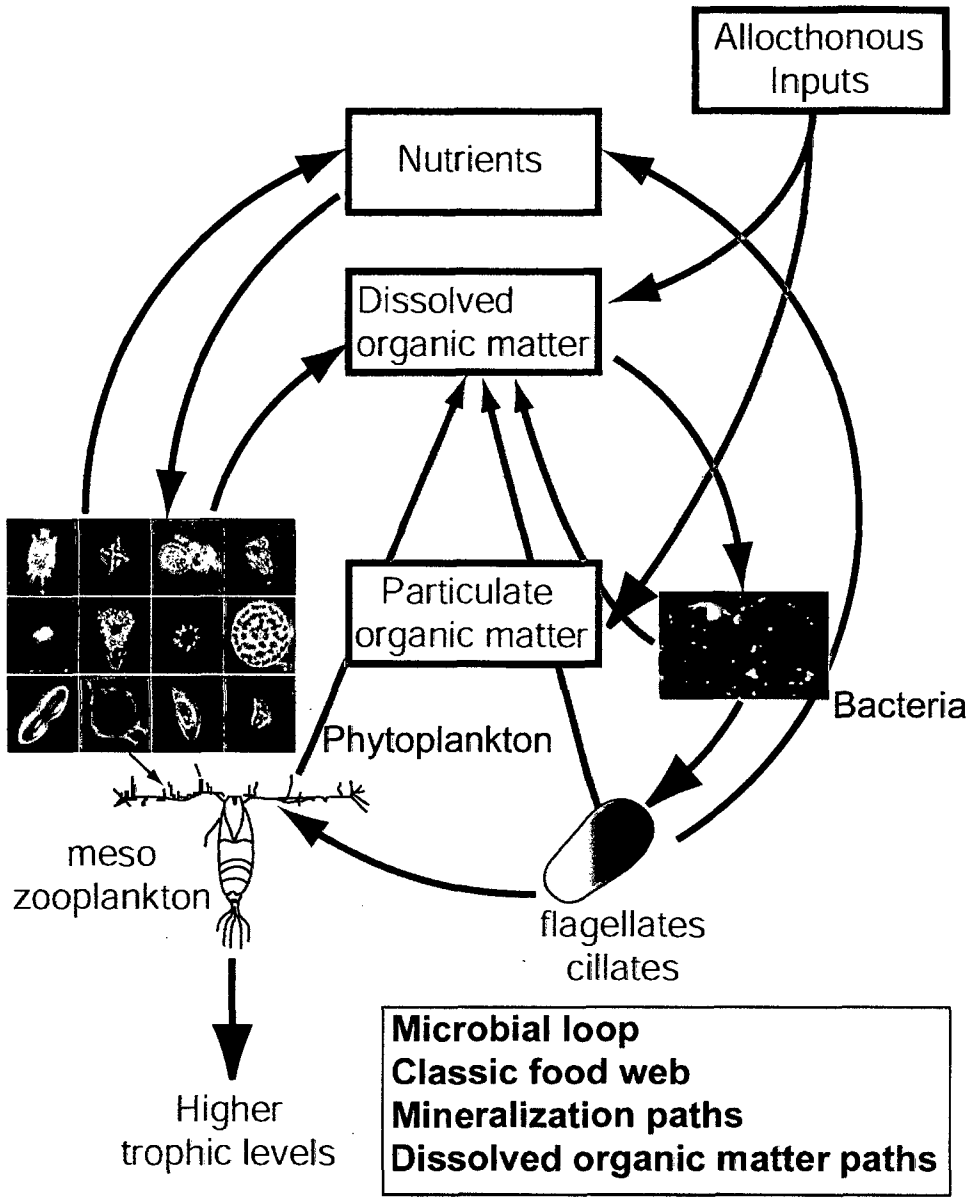


Fig. 1.1 Production of organic matter in aquatic environment and its fate in the water column. Role of microorganisms in the "microbial loop" inserted into the classical food-web. Here the dissolved and particulate organic matter, which is partly derived by the phytoplankton and partly produced by the feeding activities of the zooplankton (phytoplankton debris, excretions, fecal droppings), allochthonous inputs (terrestrial plants and soil) is efficiently scavenged by the heterotrophic bacteria.
(from-http://www.com.univ-mrs.fr/IRD/atollpol/commatoll/ukbactpl.htm).

DOC is used as carbon source by heterotrophic prokaryotes and energy is stored in the form of cell biomass. This cell biomass can re-enter the marine food chain after grazing by protozoans via the "microbial loop" (Fig. 1.1) (Azam et al., 1983). Heterotrophic bacteria play an important role in the marine carbon cycle, because 50% of marine primary production is channelled via the microbial loop (Azam, 1998; Kirchman et al., 2009).

1.2 Carbohydrates and their building blocks

Carbohydrates are the polyhydroxy aldehyde or polyhydroxy ketone compounds. Carbohydrates contain carbon, hydrogen and oxygen in the ratio of 1:2:1, with the general formula $Cn(H_2O)n$, where n = number of carbon atoms. Carbohydrates are classified into four major groups: monosaccharides, disaccharides, oligosaccharides and polysaccharides.

1) Monosaccharides are building blocks of carbohydrates. They are simple sugars and classified as aldoses and ketoses, which contain aldehyde and keto groups, respectively. The number of carbon atoms in the molecules is indicated by the name i.e. trioses, tetroses, pentoses, hexoses and heptoses. Most monosaccharides contain 5 or 6 carbon atoms and are named pentoses ($C_5H_{10}O_5$) (ribose, xylose, arabinose, etc) and hexoses ($C_6H_{12}O_6$) (glucose, galactose, mannose, etc) (Fig. 1.2a). In marine or estuarine environment dissolved monosaccharide concentrations are generally low.
2) Disaccharides consist of two monosaccharide molecules such as sucrose (common sugar contain one fructose and one glucose

molecules), lactose (milk sugar contain one glucose and one galactose molecules) and maltose (malt sugar (glucose), formed during the degradation of starch).

3) Oligosaccharides are carbohydrates which yield three to twenty monosaccharides units, and are relatively less common carbohydrates than the other groups of polymers. Oligosaccharides are trisaccharides raffinose (consists of galactose, glucose and fructose found in plants).
4) Polysaccharides are defined as high molecular weight carbohydrates containing up to thousands of monomeric units connected to one another by a covalent bond called as glucosidic bond. The most important polysaccharides that serve as energy reserve are starch, glycogen, cellulose and chitin. Glucose is common unit in starch and glycogen. Polysaccharides are further divided into two groups depending on monosaccharide composition. 1) Homopolysaccharides, 2) Heteropolysaccharides. Based on their function polysaccharides are further classified as

   i) Exopolysaccharide

   ii) Storage polysaccharides (eg. starch, glycogen).

   iii) Structural polysaccharides (eg. cellulose, chitin).

The presence of the hydroxyl groups allows carbohydrates to interact with the aqueous environment and to participate in hydrogen bonding, both within and between chains. Derivatives of the carbohydrates can contain nitrogens, phosphates and sulfur compounds. Carbohydrates can combine with lipid to form glycolipids and with protein to form glycoproteins.

Depending on their chemical composition carbohydrates are further divided into three groups-

1) Neutral carbohydrates eg. glucose, galactose, etc. (Fig. 1.2a)
2) Acidic carbohydrate (Fig.1.2b)
3) Amino sugars (-NH_2 group), eg. glucosamine, galactosamine etc (Fig1.2b)

Neutral sugars

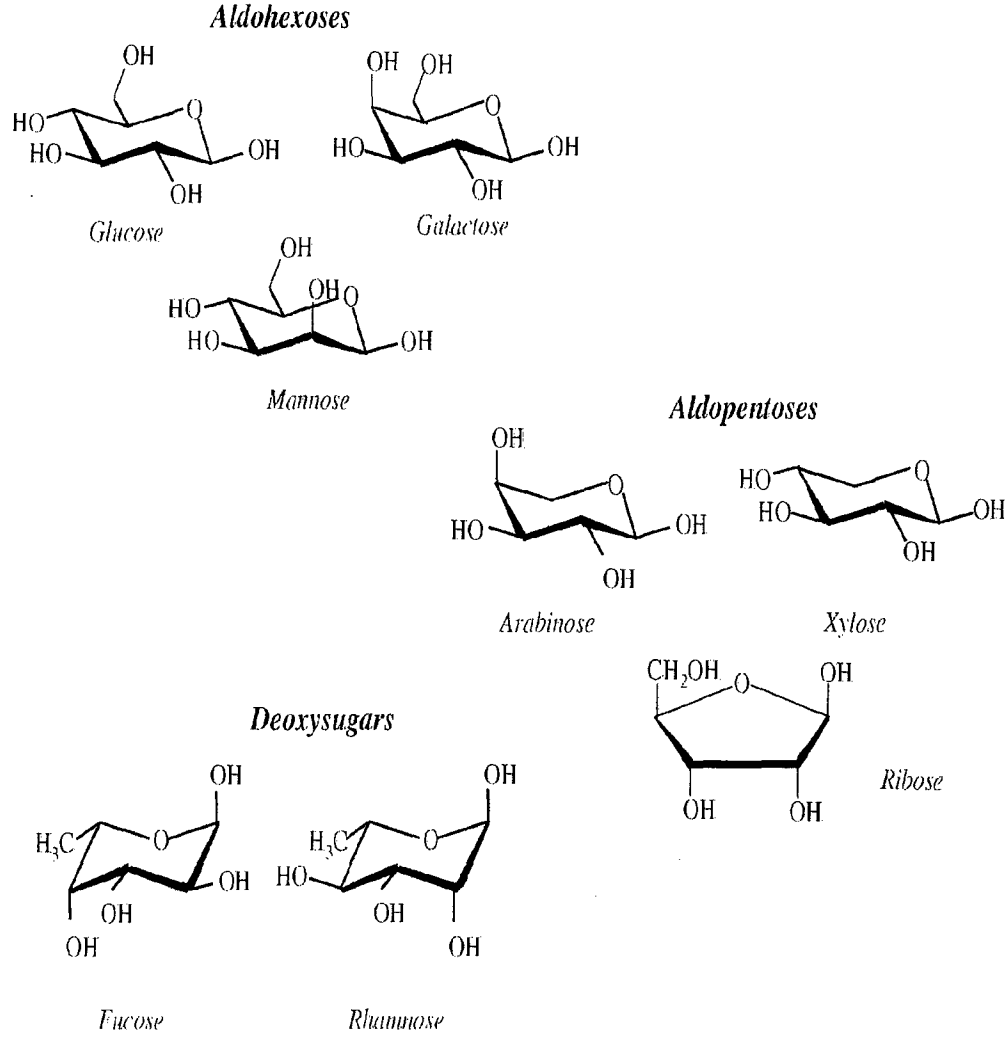


Fig. 1.2a Examples of some common monomers aldohexoses, aldopentoses and deoxysugars found in seawater, particulate organic matter and sediments after acid hydrolysis (Panagiotopoulos and Sempere, 2005).

Acidic carbohydrates and amino sugars

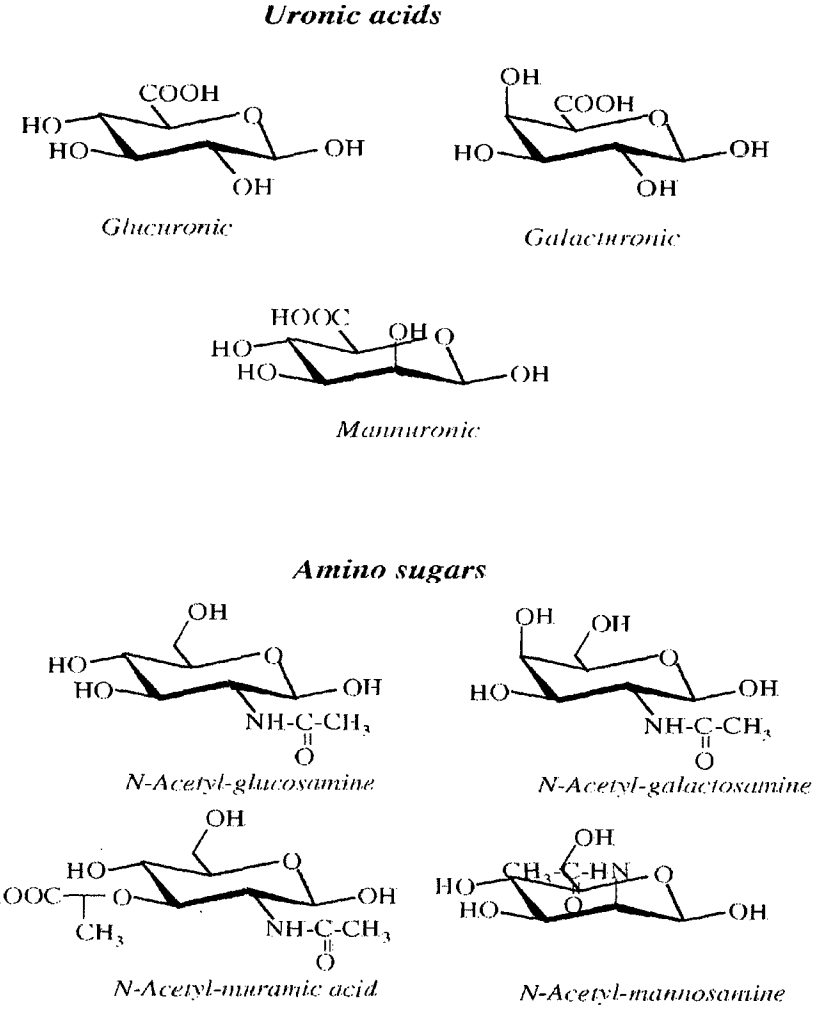


Fig. 1.2b Examples of some common uronic acids and amino sugars found in seawater, particulate organic matter and sediments after acid hydrolysis (Panagiotopoulos and Sempere, 2005).

1.3 Analytical methods for carbohydrate measurement in natural waters

Carbohydrates have often been measured using colorimetric methods, in which sugars reacts with various chemical compounds to form a colour substance that can be measured by spectrophotometry (light absorption). The

weakness of most of the colorimetric methods is that their sensitivity is too low to measure natural concentrations of free dissolved carbohydrates. The colorimetric methods only react with free monomers. This means that the concentrations of polysaccharides and complex carbohydrates must be hydrolyzed using dilute HCl or concentrated H_2SO_4 method (Pakulski and Benner, 1994; Bhosle et al., 1998) to their monomer substances to be quantified. Most commonly used methods for total sugar estimation are phenol-sulphuric acid (PSA), 3-methyl-2-benzo thiazoline hydrazone hydrochloride (MBTH), and 2,4,6-tripyridyl-s-triazine (TPTZ). Because of their high sensitivity, MBTH and TPTZ methods are commonly used to estimate dissolved carbohydrates in natural seawater and estuarine waters. Of these two, the TPTZ method is preferentially used because it is simple and less time consuming.

Concentrations of free monosaccharides as well as monosaccharide composition of polysaccharides (after hydrolysis) are measured using gas chromatography (GC) and high-performance liquid chromatography (HPLC). The GC method requires several derivatization steps, while the HPLC method with pulsed amperometric detection (PAD) can produce a relatively fast and direct quantification of mono and disaccharides in natural samples. However, desalting of marine samples is of concern as it involves losses.

1.4 Sources of carbohydrates in natural waters

In aquatic environments carbohydrates are mostly produced by photosynthetic organisms such as microalgae and aquatic plants. Bacteria

release extracellular carbohydrates. Moreover, bacterial cell wall contains several different types of carbohydrates. Fungi and insects cell wall and external skeleton (exoskeleton) contain amino sugars that can be converted to free ammonium and glucose molecule. Carbohydrates are also released in to waters due to sloppy feeding of zooplankton, and microbial degradation of organic matter. River run off and physical transport of terrestrial organic matter are some of the important sources of carbohydrates in marine waters. Phytoplankton, bacterial cells, marine suspended and sinking particles, and terrestrial organic matter are important sources of particulate and sedimentary carbohydrates in aquatic environments.

1.5 Distribution of carbohydrates in aquatic environments

The first study on carbohydrate distribution in the coastal waters of the Gulf of Mexico was carried out by Collier et al. (1950). In later years extensive studies have been carried out on the distribution and cycling of dissolved carbohydrates (Lewis and Rakestraw, 1955; Handa, 1970; Burney et al., 1981; Pakulski and Benner, 1994; Bhosle et al., 1998; Hung et al., 2005; Myklestad and Borsheim, 2007; Khodse et al., 2010) and the particulate carbohydrates (Parsons and Strickland, 1962; Handa, 1970; Bhosle and Wagh, 1989; Panagiotopoulos and Sampere, 2005, Khodse et al., 2007, 2009; Hung et al., 2009) and sediments (Cowie and Hedges, 1984; Bhosle and Dhople, 1988; Burdige et al., 2000; Khodse et al., 2008; He et al., 2010) samples in the marine environments.

1.5.1 Dissolved carbohydrates

Earlier investigations have shown that ~90% of carbohydrates in the marine waters are in solution. In aquatic environments, the content of dissolved carbohydrates varied from 0.6 μM C to 276.1 μM C in surface waters (Engbrodt and Kattner, 2005; Yang et al., 2010) and 180 μM C to 2400 μM C for sediment pore water (Burdige et al., 2000). Dissolved carbohydrates accounted for a large fraction (~10 to 85%) of DOC in seawater (Pakulski and Benner, 1994; Hung et al., 2005; Yang et al., 2010; Khodse et al., 2010), pore waters (Burdige et al., 2000) and ultrafiltered DOC (Amon and Benner, 2003).

Carbohydrates distribution depends on several factors such as phytoplankton and bacterial abundance (Hung et al., 2003, 2005; Khodse et al., 2007), microbial activity (Hung et al., 2005), oxygen concentration (Pakulski and Benner, 1994; Bhosle et al., 1998; Hung et al., 2005) and external terrestrial inputs from river (Gueguen et al., 2006). Distribution of carbohydrate is also influenced by the relative distribution of storage and structural carbohydrate. Storage carbohydrates are preferentially utilized by heterotrophic bacteria. Generally, carbohydrate concentrations vary with depth due to the differences in production and utilization and accumulation of carbohydrate. Earlier investigations on carbohydrates indicated that dissolved carbohydrates decreased from surface water to greater depth in sea water (Pakulski and Benner, 1994; Bhosle et al., 1998; Amon and Benner, 2003; Nieto-Cid et al., 2004; Myklestad and Borsheim, 2007), pore water (Burdige, 2000). In contrast, in some marine environments, carbohydrates increased with increasing water depth (Pakulski and Benner, 1994; Bhosle et al., 1998).

This increase was suggested to be due to the presence of low oxygen concentrations (oxygen minimum layer in sea) and relatively higher abundance of structural carbohydrate.

1.5.2 Particulate carbohydrates

Particulate carbohydrates serve both as structural and storage components in marine and terrestrial organisms. Earlier reports on particulate carbohydrate distribution indicated higher concentrations in river and coastal water as compared to oceanic waters. The content of carbohydrates in suspended material of the surface waters in aquatic environments varies between traces to 55.7 μM C for tropical Ganges River estuary (Ittekkot et al., 1985). In aquatic environment, carbohydrates comprise more than 40% dry wt. of bacteria and 75% dry wt. of vascular plants, 2-4% dry wt. of marine zooplankton, 20-70% dry wt. of marine algae, 10-35% of particulate organic carbon (POC) (Romankevich, 1984; Bhosle et al., 1992; Khodse et al., 2007), and 13% of sedimenting organic carbon (Tanoue and Handa, 1987).

Distribution of carbohydrates in water column is influenced by the chlorophyll *a* concentrations and bacterial abundance. For example, carbohydrates had significant positive correlation with chlorophyll *a* in the waters of Dona Paula Bay (D'souza and Bhosle, 2001), for Galveston Bay (Hung et al., 2001) and for northern Indian Ocean (Khodse et al., 2007). Moreover, carbohydrates had significant positive correlation with bacteria for northern Indian Ocean (Khodse et al., 2007). These results suggest that

phytoplankton and bacteria are major factors that control or influence the distribution of particulate carbohydrates in marine waters.

1.5.3 Carbohydrates in marine sediments

For marine surface sediments (0-5 cm), carbohydrate concentrations ranged from 3.3 μmol C in Ligurian Sea (Fabiano et al., 1995) to 1533 μmol C in Chesapeake Bay (Burdige et al., 2000). Carbohydrate carbon accounted 3-26% of sedimentary organic carbon (Burdige et al., 2000; Khodse et al., 2008; He et al., 2010). Greater concentrations of carbohydrates occur in shallow water sediments of high biological productivity waters whereas, lower concentrations occur in the slowly accumulating sediments.

1.6 Factors influencing carbohydrates distribution

Carbohydrate concentrations in water, particulate matter and sediments tend to decrease with increasing water column or sediment core depth and distance from shore because of the longer residence time of organic particles and their rapid utilization by the situ organisms during its transport from the euphotic layer to greater depths (Handa and Tominaga, 1969; Ittekkot et al., 1982; Bhosle and Wagh, 1989; Khodse et al., 2007; Hung et al., 2009). Such selective utilization of labile carbohydrates (storage polysaccharides) results in the accumulation of relatively less degradable structural polysaccharides (refractory sugars) as major components of carbohydrates in deep oceanic waters, particulate organic matter and marine sediments (Tanoue and Handa, 1987; Cowie and Hedges, 1992; Burdige et al., 2000). Structural

carbohydrates are relatively more refractory in nature and therefore, are more likely to leave an imprint on the geological record in sediment.

1.7 Uptake of carbohydrates by bacteria

In aquatic environments, heterotrophic organisms can utilize different monosaccharides for production of energy and synthesis of new cell material. Bacteria are efficient in assimilating monosaccharides, even at very low concentration, significantly affecting the distribution of free monosaccharides in natural waters. The ability of bacteria to assimilate glucose has previously been used as an indicator of bacterial activity in natural waters. This approach was used for comparison of different environments, although it did not indicate the actual bacterial growth rate (Jorgensen, 1990). Bacterial uptake rate was determined using the radioactively labelled tracers of monosaccharides in many aquatic environments. These uptake rates were further related to the bacterial production to determine the nutritional importance of carbohydrates to bacterial metabolism. For example, uptake of radioactively labelled glucose has been found to vary from 0.4 to 75 nmol l^{-1} h^{-1}, and sustain from 1% to 46% of the bacterial carbon demand (Jorgensen, 1990). In natural waters, turnover rate constants for glucose were much higher than for galactose, fucose, mannose and glucosamine (Bunte and Simon, 1999), and fructose (Jorgensen and Jensen, 1994). Large variability in uptake rates of carbohydrates is probably due to availability of other compounds (eg. amino acids), or composition of total carbohydrates was unattractive or required special enzymes to degrade carbohydrates. Second

possibility is that different bacterial species may differ in their preference for carbohydrates as nutrients. Earlier investigations suggested that carbohydrates provide 22 to 71% of carbon required for bacterial growth in natural waters (Bunte and Simon, 1999; Hanisch et al., 1996). It indicates that carbohydrates are most important carbon sources for bacteria. For example, dissolved carbohydrates utilization rates of 1.5 μg C $l^{-1}h^{-1}$ for the Atlantic waters (Burney, 1986) and 2.75 μg C $l^{-1}h^{-1}$ for a mesotrophic Danish lake have been reported (Jorgensen and Jensen, 1994). Moreover, carbohydrate utilization rates varied seasonally (0.47 to 3.43 μg C $l^{-1}h^{-1}$) (Hanisch et al., 1996).

DOM studies have shown that substrate quality and composition play an important role in the recycling of organic matter. Carbohydrates and amino acids are preferentially utilized during microbial decomposition of marine organic matter (Skoog and Benner, 1997, Lee et al., 2000, Amon et al., 2001), and serve as useful indicators of diagenetic history of organic matter (Cowie and Hedges, 1994, Dauwe and Middelburg, 1998).

In aquatic environments, utilization of DOM by heterotrophic bacteria depends on biochemical composition, molecular size, inorganic nutrient concentrations, photochemical transformation and temperature (Amon and Benner, 1996; Rosenstocks et al., 2005; Abboudi et al., 2008). Saunders (1976) suggested that low molecular weight (LMW) DOM (< 1kDa) was utilized faster as compared to the high molecular weight (HMW) DOM (>1 kDa) because LMW DOM (<1 kDa) substances are less complex and can passively pass through the cell membrane without any expense of energy.

Whereas, HMW DOM (>1 kDa) substances are more complex and in many cases need to be externally degraded to simpler compounds by exoenzymes and then transported across the cell membrane by simple diffusion or by a carrier protein. Similar observations have been reported by Axmanova et al. (2006). In contract, DOM size-fractionation experiments conducted by Amon and Benner (1994, 1996) were helpful to understand the cycling of organic matter in the ocean environment. The yields of hydrolyzable neutral sugar and amino acids decreased with increasing decomposition and diagenetic alteration (Cowie and Hedges, 1992). The depth related trends in the size, composition and bioreactivity of DOM in the ocean indicate that most of the biorefractory DOM in the deep ocean exists as LMW DOM (<1 kDa). It has also been reported that DOM >1 kDa or 10 kDa can support consistently higher bacterial growth than LMW DOM (<1 kDa) fraction (Volk et al., 1997). Rochelle-Newall (2004) suggested that bioavailability of LMW DOM and HMW DOM factions varied seasonally. Moreover, bacterial utilization of size fractionated DOM in Dona Paula Bay waters suggested that LMW DOM (>10 to 30 kDa) fraction was labile and supported higher bacterial growth than HMW DOM (>30 kDa to 0.22 μm). It appears that in oceanic environment HMW DOM (>1 kDa) was preferentially degraded by bacteria because phytoplankton is a major source of DOM. However, This may not be the case for estuarine environment where DOM is mostly derived from various sources (phytoplankton, terrestrial plants, humic rich soil) (Rosenstock et al., 2005).

1.8 Production of carbohydrates by microorganisms

Algae and bacteria produce extracellular carbohydrates as they enhance their growth and survival in natural environments (Costerton, 1999; Biddanda and Benner, 1997). Extracellular carbohydrates are constituents of marine particles, sediments (Cowie and Hedges, 1984) and marine DOC (Wang et al., 2006). Phytoplankton release DOC during primary production (Nagata, 2000). Carbohydrates account for a large proportion of DOC (Biddanda and Benner, 1997; Leandro et al., 2003). Carbohydrate production by diatoms under laboratory conditions has been demonstrated in numerous studies (Myklestad, 1974; Bhosle et al., 1993, 1995; Leandro et al., 2003). Such studies indicate that type, quantity and composition of carbohydrates are influenced by diatom species (Myklestad, 1974; Leandro et al., 2003), nutrient status, growth phase (Myklestad et al., 1972; Handa, 1969; Urbani et al., 2005) and pH of culture medium (Thornton, 2009). Production of uronic acids and amino sugars is also influenced by growth condition of diatoms (Khodse and Bhosle, 2010). Production of extracellular carbohydrate by diatom increased under high light and low nutrient conditions (Staats et al., 2000). Bacteria and phytoplankton produce greater amounts of cellular and extracellular carbohydrates during the stationary phase of growth and under nutrient depleted condition in the growth medium or in natural waters (Myklestad et al., 1972; Urbani et al., 2005). For example, increase in carbohydrate concentrations have been reported at the end of the phytoplankton blooms in the oceanic/coastal environments (Ittekkot et al., 1981; D'souza and Bhosle, 2001) and in the laboratory studies (Myklestad, 1974; Bhosle et al., 1993, 1995; Leandro et al., 2003). Moreover, nutrient

status and growth phase of microorganisms influence the quality and chemical composition of the carbohydrates produced (Decho, 1990). Furthermore, each bacterial and diatom species may produce unique polysaccharides (Hogaland et al., 1993; Bhosle et al., 1995; Carvalho and Fernandes, 2010).

1.9 Storage and structural polysaccharides

Carbohydrates serve as both structural and storage compounds in marine and terrestrial organisms. Excess photosynthesis may lead to accumulation of storage carbohydrates (Myklestad and Haug, 1972). Storage carbohydrates of most algae are α- or β –glucans. Chrysolaminaran is most abundant type of storage carbohydrate in marine phytoplankton (Chiovitti et al., 2004). Storage carbohydrates are produced in light condition and serve as internal energy for carbon reserve. At low light condition or at night storage, carbohydrates are used for maintaining cell metabolism and protein synthesis (Granum and Myklestad, 2001).

In aquatic environments, structural polysaccharide cellulose is found in plants, phytoplankton and bacterial cell wall. These polysaccharides are generally present in polymeric forms wherein the monomer residues are bound to each other by glycosidic bonds. These glycosidic bonds are digested by hot acid digestion but are fairly stable in neutral and alkaline solutions. Ester linkages, which are involved in binding of polysaccharides to other organic molecules, are easily destroyed by alkaline treatment. Carbohydrate and proteins are bound to each other by *O*-glycosidic bonds

which are also hydrolysed under alkaline condition (Collins and Ferrier, 1995). Alkali-soluble polysaccharides are involved in initial attachment of the diatom cells to surface (Bhosle et al., 1993). The hydrogen and van der Waals bonds, which bind the organic macromolecules with each other and with cell-surfaces, can be dissociated by heating. Therefore, treatment with hot water or dilute acid or dilute alkali (Hitchcock, 1977) and heat (Handa and Tominaga, 1969) often have been applied to selectively extract storage and structural polysaccharides from diatoms, natural suspended particulate matter and sediments without significant destruction of polymer structure (Handa and Tominaga, 1969; Handa and Yanagi, 1969; Haug et al., 1973; Hitchcock, 1977; Bhosle and Dhople, 1988; Khodse et al., 2008).

1.10 Uronic acids

Uronic acid is an aldehydic acid that differs from an aldose by having a terminal carboxyl carbon opposite the C-1 carbonyl carbon. Uronic acids are commonly found in animals, terrestrial plants, algae, and bacteria (Walter and Hedges, 1988; Bergamaschi et al., 1999; Hung et al., 2003; Khodse and Bhosle, 2010). They are also present in dissolved organic matter, particulate matter, and marine sediments (Bergamaschi et al., 1999; Hung et al., 2003, 2005; Khodse et al., 2007, 2008, 2010). They serve both ion-regulation and structural roles. Many microorganisms produce acidic polysaccharides (Costerton, 1984; Decho, 1990). These carbohydrate polysaccharides may contain sulphate, pyruvate, acetate, and acidic group such as uronic acids and amino sugars (Decho, 1990; Kaiser and Benner, 2000; Guo et al., 2002;

Khodse et al., 2008). Some commercially important plant structural polysaccharides, such as pectin and alginic acid are almost entirely composed of glycosidically bound uronic acids. Bacteria contain a wide variety of uronic acids as major components of structural and extracellular polysaccharides. These compounds are involved in several marine environmental processes including formation of humic substances (Santschi et al., 1998), removal of toxic metals, detoxification of toxic chemicals (Ford et al., 1987; Hung et al., 2003; Bhaskar and Bhosle, 2005; Perez et al., 2008), and production of macroaggregates (Alldredge and Silver, 1988). Uronic acids play an important role in bacterial adhesion (Jain and Bhosle, 2009), and biofilm formation (D'souza and Bhosle, 2003; Bellinger et al., 2009; Khodse and Bhosle, 2010). Uronic acids as compared to neutral sugars are resistant to bacterial degradation. Thus, uronic acids are of ecological significance and play important role in biogeochemical cycling of organic matter in the aquatic environments.

1.11 Amino sugars

Amino sugars are unusual carbohydrates in that they contain nitrogen. The amino sugars such as glucosamine (GlcN), galactosamine (GalN), mannosamine (ManN), and muramic acid (Mur) are detected in marine and estuarine samples (Fig. 1.2b). These amino sugars are found in chitin, peptidoglycan, polysaccharides, glycoproteins, and glycolipids (Benner and Kaiser, 2003; Bahulikar and Kroth, 2008). Chitin is the second most abundant polymer on earth (Cohen, 1987) and the most abundant biopolymer in the

ocean (Benner and Kaiser, 2003). It is a structural polymer of the amino sugar N-acetyl-glucosamine and is produced by a large variety of marine organisms like copepods, algae, bacteria and fungi (Muzzarelli, 1977; Benner and Kaiser, 2003). Peptidoglycan is an important structural component of prokaryotic cell walls (Brock et al., 1994) and is composed of N-acetylglucosamine, N-acetylmuramic acid, and L- and D-amino acids (Sharon, 1965; Dittmar et al., 2001). Muramic acid has only been detected in bacterial cell walls (Sharon, 1965) and can reach concentrations up to 10% of the whole bacterial biomass (Moriarty, 1975). Recently, a few studies have been carried out on the amino sugar cycling in marine waters. Amino sugars have been employed as indicators of organic matter production and degradation (Benner and Kaiser, 2003; Davis and Benner, 2005; Niggemann and Schubert, 2006; Tremblay and Benner, 2006; Davis et al., 2009).

In soil science, amino sugars are well investigated, and have been used as biomarkers for microbial residues (Zhang and Amelung, 1996; Amelung, 2001; Turrion et al., 2002; Amelung, 2003; Glaser et al., 2004). Distribution of amino sugars has been reported in dissolved and particulate organic matter and sediments from some marine environments (Kemp and Mudrochova, 1973; Niggemann and Schubert, 2006; Dauwe and Middelburg, 1998; Jennerjahn and Ittekkot, 1999; Gupta and Kawahata, 2000). These studies were useful to assess sources and degradation state of organic matter (Mimura and Romano, 1985; Müller et al., 1986; Jennerjahn and Ittekkot, 1999; Gupta and Kawahata, 2000; Benner and Kaiser, 2003; Niggemann and Schubert, 2006). Muramic acid is a useful biomarker to

assess the bacterial contribution to dissolved, particulate, and sedimentary organic matter (Kaiser and Benner, 2008; Tremblay and Benner, 2009; Bourgoin and Tremblay, 2010).

1.12 Role of carbohydrate in aquatic environments

1.12.1 Food web

In aquatic systems, organic matter containing carbohydrates is a well known energy source for microbial consumer animals (Decho, 1990; Rolff and Elmgren, 2000; Bhaskar and Bhosle, 2006). Microbial exopolysaccharides (EPS) are present in aquatic environments and they are closely associated with the microbial flora, detritus material, and sediments. Consumer animals while feeding on bacteria, phytoplankton, detritus material, and sediment, will coincidently consume exopolymers, and their adsorbed DOM (Decho, 1990; Nichols et al., 2005; Arnous et al., 2010). This implicates the importance of EPS as a food source for aquatic animals. Adsorption of labile materials (sugars and amino acids etc, metals) by microbial exopolymers provides essential elements (C,N,S, etc) and micronutrient to the higher trophic organisms (Bhaskar and Bhosle, 2006; Ferry et al., 2009).

1.12.2 Metal cycling

In aquatic environments, EPSs secreted by bacteria, fungi and algae are involved in heavy metal removal due to extensive adsorptive capacity (Veglio et al., 1997; Pagnanelli et al., 2000; Bhaskar and Bhosle, 2006). The adsorption of heavy metal by polysaccharides is a metabolism-independent

process, and is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS (Hung et al., 2003; Passow et al., 2006). A significant fraction of dissolved trace metals is associated with colloidal organic carbon (Honeyman and Santschi, 1992; Gue et al., 2002), especially with polysaccharides (Quigley et al., 2002; Hung et al., 2005; Quiroz et al., 2006). Polysaccharide-metal complexes are used as source of food by many organisms thereby transferring these metals to higher trophic levels in the food chain. During the sedimentation process, metal ions bind to polysaccharides and are transported to bottom sediments (Nealson, 1983). Such a process is involved in the accumulation of metal pollutants in deep waters (Decho, 1990). In contrast, accumulated metals undergo microbial transformations and transferred back to water column. Thus, polysaccharides play a key role in the cycling of trace metals (Passow et al., 2002).

1.12.3 Biofilm formation

Biofilms are readily formed on most submerged surfaces such as rock, detritus, and variety of man-made surfaces (Ship hulls, water pipes, etc.) as well as internal surface of human body. Biofilm is a complex aggregation of microorganisms growing on a solid substrate. While attaching to surfaces microorganisms produce EPS which are generally rich in carbohydrates (Jain and Bhosle, 2009). EPS biopolymers are highly hydrated and form a matrix, around the biofilm cells. EPSs in biofilms are known to perform several functions including the protection and maintenance of biofilms.

1.12.4 Provides protective barrier to the cell

Exopolymer slime and capsular EPS provides a matrix around biofilm cells thereby EPS may provide protection to cells within the aggregates (Decho, 1990; Decho and Lopez, 1993; Hoagland et al., 1993; Bahulikar and Kroth, 2008). Boyle and Reade (1983) observed that changes in pH and salinity over a wide range had little effect on the viscosity and stability of EPS produced by marine bacteria. Such results suggest that these polymers provide a buffer against sudden changes in environmental conditions in the natural environments (Dudman, 1977). EPS determine the immediate conditions of life of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability (Flemming et al., 2002). For example, EPS prevents desiccation of biofilms in some natural environments. EPS may also contribute to the antimicrobial properties of biofilms and prevent the mass transport of antibiotics or biocides through the biofilm and probably by binding directly to these agents (Donlan, 2002).

1.12.5 Ecosystem stability

Bacteria and microalgae have been intensively studied in the aquatic environments. This is because of their conflicting importance in the production and mineralization of organic matter and inorganic compounds and their roles as consumer of food resources and the cycling of carbon and nitrogen (Pomeroy, 1974; Hedges et al., 1992; Arnous et al., 2010). Biological production, sediment erosion and human activities and transport are a

governing factor in the ecological and commercial health of aquatic ecosystems. This important ecosystem function has mainly been linked to microalgae ("ecosystem producer") and their associated EPS. Yet little is known about the impact of bacterial assemblages and how their varying interactions with microalgae affect the overall biostabilization potential of the ecosystem. The monomeric labile compounds (sugars) are important energy source and utilised faster by bacteria. DOM is the largest pool of organic matter in aquatic environments (Wetzel, 1992). Terrestrially derived humic acids are the major constituent of DOM pool (Thurman, 1985), and these polymeric molecules have been considered both chemically and biologically refractory (Guo and Santschi, 2000; Hansell and Carlson, 2007). Subsequently, some studies have shown that humic molecules are susceptible to photolytic (Opsahl and Benner, 1998; Rosenstock et al., 2005) and microbiological degradation (Volk et al., 1997; Carlson et al., 1999; Rosenstock et al., 2005), and may thus contribute to the aquatic energy flow. The ultimate sources of all organic compounds, including monomers, are autotrophic organisms, especially photosynthetic plants. Moreover, physical, biological and chemical, processes in water column affect the microbial activity and carbohydrate utilization in surface waters described in chapter 4A. These environmental factors may play an important role in organic matter cycling within the ecosystem.

1.12.6 Importance of marine carbohydrate as biogeochemical indicators

Monosaccharide composition of neutral carbohydrates, uronic acids and amino sugars are useful tools for identifying the sources of carbohydrates (Cowie and Hedges, 1984; Ittekkot et al., 1984; Aluwihare et al., 1997; Bergamaschi et al., 1999; D'souza and Bhosle, 2001; Benner and Kaiser, 2003; Amon and Benner, 2003; Fernandes et al., 2006; Khodse et al., 2007, 2008). The relative abundance of the individual sugars and sugar ratios in a sample has been used to distinguish terrestrial, marine, siliceous and carbonaceous inputs in the marine environments (Cowie and Hedges, 1984; Ittekkot et al., 1984; D'souza and Bhosle, 2001; Khodse et al., 2007, 2008). For example, the ratio rhamnose + fucose : arabinose + xylose is useful tool to identify the sources of organic matter in marine environments. The high (> 0.5) ratio indicates the presence of marine organic matter derived from marine bacteria and phytoplankton. The glucose/ribose ratio >20 has been suggested to indicate terrestrial source (Mopper, 1973; Ittekkot and Arain, 1986). Ribose and fucose can be used as diagnostic parameters to separate marine and terrestrial carbohydrate sources. Ribose and fucose are major component in plankton and bacteria and minor in terrestrial plants (Cowie and Hedges, 1984). Ribose plus fucose mol% (glucose free basis) >10 indicates the marine sources. The mannose/xylose (Man/Xyl) ratio has been used to identify the angiosperm wood form gymnosperm wood and non-woody tissues, grasses, phytoplankton, bacteria and zooplankton (Cowie and Hedges, 1984). Arabinose plus galactose is useful to differentiate wood tissues from non-woody tissues (Cowie and Hedges, 1984).

Similarly, amino sugars ratios are also indicators of the source of organic matter. For example, GlcN/GalN ratio has been utilized to identify the sources of organic matter, with high values (> 4) indicating the abundance of chitin containing zooplankton, and constant and low values (< 3) suggest the abundance of bacteria (Muller et al., 1986; Gupta and Kawahata, 2000: Benner and Kaiser, 2003; Fernandes et al., 2006; Niggemann and Schubert, 2006; Kaiser and Benner, 2008; Tremblay and Benner, 2009). Peptidoglycan is the only source of muramic acids (Mur). Low concentration of Mur and high and variable ratio of GlcN/Mur suggests that peptidoglycan remnants are minor constituents in dissolved, particulate and sedimentary organic matter (Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Klauser and Schubert, 2007; Kaiser and Benner, 2008). Mur concentration has been used to estimate bacterial numbers in particulate organic matter and marine sediments (King and White, 1977; Moriarty, 1977; Mimura and Romano, 1985; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Kaiser and Benner, 2008).

Furthermore, contribution of neutral carbohydrates carbon to organic carbon defined as yields provides useful information on degradation state of organic matter. Relatively high yield indicates freshly derived organic matter (Cowie and Hedges, 1994; Skoog and Benner, 1997; D'souza and Bhosle, 2001; Khodse et al., 2007, 2008). $\delta^{13}C$ values are also useful to differentiate terrestrial organic matter (average $\delta^{13}C$ = -27) from marine organic matter (average $\delta^{13}C$ = -22).

1.13 Effect of global climate change on carbohydrate production

Widespread industrialization and ever increasing number of vehicles is leading to increase in the concentration of green house gases in the atmosphere. Green house gases are hypothetically responsible for the global warming and other climatic changes. It not only affects the atmospheric conditions, but also the oceans. High amount of dissolved CO_2 in the oceans decrease the seawater pH, thus making it less alkaline (Caldeira and Wickett, 2003). The increasingly acidic marine waters can influence marine phytoplankton. Phytoplankton plays a key role in biogeochemical cycling and ecosystem functioning in oceans. Increase in acidity of sea water affected the quantum efficiency of photosystem II and carbohydrate metabolism in a planktonic diatom such as *Chaetocerosmuelleri* sp. (Thornton, 2009). Recently, Thornton (2009) observed that when *Chaetocerosmuelleri* sp. was grown in batch culture at low pH, the proportion of total carbohydrate stored within the cells decreased while greater amounts of dissolved carbohydrates were released into the growth medium. The ratio of cell carbohydrate to dissolved extracellular carbohydrate was 2.60 ± 0.26 at pH 8.2; 1.99 ±0.41 at pH 7.9, and 1.24 ±0.41 at pH 7.4.

Lu et al. (2006) studied physiological changes in *phycocyanin* (PC)-rich and *phycoerythrin* (PE)-rich *Synechococcus* strains of picocyanobacteria under atmospheric CO_2 concentrations of 350, 600 and 800 ppm in batch cultures maintained in one-liter glass flasks under a 12 hour light : 12 hour dark regime for periods of 12 days. The *phycocyanin* strain showed no significant change in carbohydrate content over the CO_2 range investigated; but the exhibited a CO_2-induced increase of 37.4% at 800 ppm CO_2. Rise in

the temperature and level of CO_2 would stimulate growth and photosynthesis (Fu et al. 2007). Kirchman et al. (2009) suggested that the heterotrophic activity will increase with increase in temperature. In the polar region climate change may be responsible for the shift in the heterotrophic production/ primary production energy flow through the aquatic food web.

The shift in carbon allocation to different pools by diatoms at low pH may have implications for the ecosystem function, the efficiency of the biological carbon pump, and the resulting sequestration of CO_2 in the deep ocean (Riebesell et al., 2007). Less carbohydrate stored within the cells, and a greater production of low-molecular-weight dissolved extracellular carbohydrates at low pH, will result in less particulate organic carbon in the water column, potentially reducing the efficiency of the biological carbon pump during diatom blooms.

Aim and Scope of the present research

There have been several studies on the distribution of carbohydrates in marine waters, and production of dissolved carbohydrates by marine phytoplankton and their consumption by marine heterotrophic bacteria. However, most of these studies on dissolved and particulate carbohydrates have been carried out in temperate, Arctic and Antarctic waters. In contrast, little is known about the production of carbohydrates by tropical microorganisms and the distribution and cycling of carbohydrates in tropical marine and estuarine waters. Moreover, on global basis, not much is known about the distribution and fate of carbohydrate species such as uronic acids

and amino sugars in marine/estuarine environments. Carbohydrate polymers are also involved in biofilms. Therefore, in order to understand the biofilm process, there is a great need to identify the differences in carbohydrates and other constituents in planktonic and biofilm cells of diatoms. In view of these observations, the goals of this research were to understand the production, distribution, sources, and fate of carbohydrates in tropical marine environments and also to evaluate its importance in biofilm process. In order to achieve these goals, the research presented in this thesis was planned with the following objectives-

1) assess the production and biochemical characterization of carbohydrates produced by microorganisms such as marine diatom and bacteria, 2) identify the major differences in the concentration and composition of carbohydrates, uronic acids, and amino sugar in planktonic and biofilm cells of marine diatoms, 3) evaluate the distribution of carbohydrates, uronic acids and amino sugars in the Mandovi estuary, and identify some of the major drivers that controlled the distribution of these compounds in this estuary on the west coast of India, and 4) to assess the utilization of size fractionated dissolved organic matter including carbohydrates and uronic acid by natural population of bacteria.

Chapter 2

Production of dissolved carbohydrates and uronic acids by marine *Bacillus* spp.

2.1. Introduction

Carbohydrate polymers released in aquatic environments or growth medium are defined as extracellular carbohydrates (EPS). These carbohydrate polymers help microbial communities to tolerate extremes of temperature, salinity and nutrient availability (Decho 1990; Nichols et al., 2005; Poli et al., 2010). Many microorganisms (bacteria, archaea, fungi and algae) are known to produce extracellular polysaccharides (Sutherland, 1972; Decho, 1990; Poli et al., 2010). Concentration and composition of EPS produced by microorganisms are influenced by carbon source, nutrient status, and growth phase (Decho, 1990; Costerton, 1999), temperature etc. (Nichols et al., 2005).

EPSs are chemically diverse and contain carbohydrates, proteins, nucleic acids and lipids (Decho, 1990; Nielsen et al., 1997; Spaeth and Wuertz, 2000; Nichols et al., 2005; Tian, 2008). The presence of proteins, nuronic acids, lipids, uronic acids, pyruvic acid, and O-methyl, O-acetyl and sulfate groups emphasizes the complex nature of bacterial EPS. EPS are known to perform several functions such as improvement of water holding capacity of soil, sedimentation of phytoplankton, biofilms formation, and detoxification of heavy metals and removal of solid matter from water column (Decho, 1990). However, little information is available on the production of different species of dissolved carbohydrates (monosaccharides, polysaccharides and acidic carbohydrates) by marine microorganisms, especially by bacteria.

In aquatic environment, bacterial numbers varied with season, and location and ranged from 10^5 to 10^8 cells ml^{-1} (Ramaiah et al., 1996; Bode et al., 2001; Khodse et al., 2010). Among the bacterial community, *Bacillus* sp. represent a diverse group of bacteria that are widely distributed in aquatic environments due to their ability to form spores and withstand a range of variable environmental conditions (Priest, 1993; Parvathi et al., 2009). *Bacillus* spp. adapt easily to diverse habitats (Priest, 1993). During the bacterial growth, up to 45% of organic carbon like glucose utilized by bacteria is converted into EPS, which is then released into the surrounding waters (Stoderegger and Herndl, 1998). In this chapter, we assessed the production, isolation and chemical characterization of carbohydrates including polysaccharides and uronic acids by four cultures of *Bacillus* sp.

2.2 Materials and Methods:

2.2.1 Source of bacterial cultures

Four bacterial cultures (CE-2, Bac, Ti-28, SS-14) were obtained from the Marine Corrosion and Material Research division (MCMRD) culture collection. All the four cultures were grown on Zobell Marine Agar (ZMA) plates and then pure cultures were transferred to ZMA slants and stored at 4 °C for further use. These bacteria were isolated from biofilm developed on stainless steel in the surface waters of Dona Paula Bay, west coast of India (details in D'Souza, 2004).

2.2.2 Bacterial growth medium

Bacterial cultures were grown in a basalt salt solution (BSS) containing (g/L); NaCl, 25.0; KCl. 0.75; $MgSO_4$. $7H_2O$, 7.0; NH_4Cl, 1.0; K_2HPO_4, 0.7; KH_2PO_4. 0.3; glucose, 1; and 1 ml of trace metal solution (Appendix-Ib). pH of the medium was adjusted to 7.5 with 1N NaOH. The medium was sterilized by autoclaving for 20 min at 121°C.

Culture conditions and inoculum prepration

Loopful of cultures from the slants were transferred into conical flasks (50 ml) containing 10 ml of above growth medium at room temperature (28 ±2 °C) on a rotary shaker at 100 rpm for 24 h. The cultures were sub-cultured twice in same medium and then used as an inoculum.

2.2.3. Bacterial Identification

Bacterial cultures were identified using both conventional physiological and biochemical tests and molecular identification method based on 16S-rDNA sequencing.

a) Conventional identification method-

Bacterial cultures were tested for gram reaction, cellular morphology, and physiological and biochemical characteristics following standard methods described in Manual of Methods in Bacteriology (1981). The culture characteristics and biochemical characteristics were compared with those given in Bergey's Manual of Systematic Microbiology (1984). Using these methods cultures could be identified up to its family. For further identification, molecular methods were used.

b) ***16S rDNA sequencing method-***

The 16S-rDNA sequencing procedure can be divided into three steps – 1) Bacterial DNA extraction, 2) gene amplification by polymerase chain reaction (PCR) and 3) DNA sequencing and analyses.

Bacterial DNA extraction

The cultures were grown overnight in 10 ml of nutrient broth (Hi Media, India) and bacterial genomic DNA was isolated using DNA isolation kit (Sigma, USA) following the protocol recommended by the supplier.

Polymerase chain reaction

The genomic DNAs of individual bacterial samples were used for PCR amplification. The 16S rDNA gene fragments were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-30) (Weisburg et al., 1991). The amplification was carried out in 25 µl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), $MgCl_2$ (25 mM), the primers 27F (10 ppm) and 1492R (10 ppm) respectively, Taq polymerase (Sigma, USA) (2U μl^{-1}) and autoclaved Milli-Q water (Millipore). The amplification was carried out for 35 cycles of 94 °C, for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72 °C for 7 min with initial 05-min denaturation at 94 °C using a PCR-Express thermal cycler (Hybaid). The DNA sequences of the PCR products were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer, Applied Biosystems, Foster City, CA, USA) using the protocol recommended by the supplier. Reaction products were sent for sequencing to GeNei™ Banglore (INDIA).

The nucleotide sequences of the 16S rDNA genes (about 1400 nucleotides each) were aligned and compared with those available in the Genebank databases of National Center for Biotechnology Information (NCBI), USA and searched for sequences similarity analysis of the 16S rDNA sequence obtained. The full 16S rDNA sequences of four bacterial cultures were deposited to the GenBank database under the accession numbers HM150643 (CE-2), HM150644 (Bac), HM150645 (SS-14) and HM150646 (Ti-28).

2.2.4 Growth curve and dissolved carbohydrate production by Bacillus spp.

To assess the growth and dissolved carbohydrate production, each bacterial culture (CE-2, Ti-28, Bac, SS-14) was grown in two 1 liter capacity conical flasks, each containing 250 ml BSS medium supplemented with 0.1% glucose as a carbon source. Cultures were grown on rotary shaker (100 rpm) at room temperature (28 ±2°C) for 144 hours. During the incubation period, a aliquot of culture broth (20 ml) was withdrawn at 4 h intervals over the first 36 h, and then at 24 h over a period of 144 h. These samples were analysed for total dissolved carbohydrate (TDCHO), free monosaccharides (MCHO), dissolved polysaccharides (DPCHO), dissolved uronic acids (DURA) and bacterial cells growth. Bacterial cells growth was monitored by measuring turbidity at 540 nm. For dissolved carbohydrate measurement, cells were removed by centrifugation at 8,000 rpm at 4°C for 15 min and the supernatant was filtered

(0.22 µm Mixed Cellulose Ester filter, Millipore), and the filtrate was used for carbohydrates and uronic acid analysis.

2.2.5 Isolation of EPS from Bacillus spp.

Large scale production of EPS by *Bacillus* spp. (CE-2, Ti-28, Bac, and SS-14) was studied in batch cultures. All four *Bacillus* sp. were grown individually in 5 liter conical flasks containing 1000 ml of BSS medium supplemented with 0.1% glucose as the sole carbon source. The medium was inoculated (1%) with 24 h grown cells of *Bacillus* sp. grown twice in the same medium. The flasks were incubated on a rotary shaker at room temperature (28 ±2°C) for six days. Flasks were removed after 6 days following the inoculation. The cells were removed by centrifugation at 8,000 rpm for 15 min at 4°C. The supernatant was passed through 0.22 µm filter to remove the any cell material. One liter filtrate was concentrated to 50 ml in stirred ultrafiltration cell (Amicon) using the 10 kDa MW cut-off cellulose membrane ultrafilter (# YM-10). The concentrated filtrate was dialysed against Milli-Q water using dialysis bags (MW cut-off of 8000) for 24 h at 4 °C. Before use, dialysis bags were washed with 0.01 M EDTA in 1% sodium carbonate followed by washing in Milli-Q water to remove the contaminating material. EPS was then precipitated by adding 4 volumes of ice-cold isopropanol and kept overnight at 4 °C to complete the precipitation process. The EPS was collected after decanting the isopropanol and partially dried. For the purification, EPS was dissolved in small volume of Milli-Q water and dialysed again against Milli-Q

water at 4°C to eliminate any low molecular weight sugars and salts using dialysis bags (MW cut-off of 8000). EPS was re-precipitated using isopropanol and collected by centrifugation at 8000 rpm for 20 min and lyophilized. The lyophilized EPS was used for chemical analyses described below.

2.2.6 Analytical methods

2.2.6.1 Carbon and nitrogen analysis

Organic carbon and nitrogen content in EPS were measured by elemental analyzer (Finnigan Flash EA-1112, Thermo electron), and 2,5, Bis-(5-tert-benzoxazol-2-yl)-thiophen (BBOT) was used as standard for carbon and nitrogen analysis. The precision of the method based on replicate measurements of a reference standard is 0.11% for carbon and 0.24% for nitrogen.

2.2.6.2 Determination of TDCHO, MCHO and DPCHO

Dissolved monosaccharide was determined by oxidation of the free reduced sugars with 2,4,6 tripyridyl -s- triazine (TPTZ) following the spectrophotometric method (Myklestad et al., 1997). The redox reactions are as follows,

$$RCHO + [2Fe(CN)_6]^{3-} + 3OH^- \longrightarrow RCO_2^- + [2Fe(CN)_6]^{4-} + 2H_2O$$

$$[2Fe(CN)_6]^{4-} + TPTZ \longrightarrow [Fe(TPTZ)_2]^{2+}$$

(violet-coloured complex)

The aldehydes of MCHO (after hydrolysis of glycosidic bonds) standard solution of reducing sugar (glucose) are oxidized at alkaline medium, wherein Fe^{3+} are reduced to Fe^{2+}. Then Fe^{+2} reacts by condensation with TPTZ to give violet-coloured complex $[Fe(TPTZ)_2]^{2+}$ which is specrophotometrically determined at 595 nm.

Total dissolved carbohydrates were determined after hydrolysis with 0.09 N HCl as described by Bhosle et al. (1998). The spent medium 5 ml was added to glass ampoule and acidified with 0.5 ml of 1 N HCl (final concentration 0.09N), flushed with nitrogen gas and sealed. The sealed sample was hydrolyzed for 20 h at 100 °C. After cooling, the sample was neutralized using 1 N NaOH. Then, 1 ml of hydrolysate was mixed in a test tube with 1 ml of 0.7 mM potassium ferricyanide (400 mg NaOH, 20 g Na_2CO_3 and 230 mg $K_3[Fe(CN)_6]$ in 1000 ml of Milli-Q water), and well mixed solution was placed in boiling water bath for 10 min. A 2 mM solution of ferric chloride was prepared by dissolving 32.4 mg of anhydrous $FeCl_3$ in 100 ml of a solution prepared by adding 16.4 g sodium acetate (anhydrous), 4.2 g citric acid and 30 g acetic acid to 1000 ml of Milli-Q water. One milliliter of this 2 mM ferric chloride and 2 ml of 2.5 mM TPTZ (2,4,6 tripyridyl -s- triazine) solution in 3 M acetic acid were immediately added and mixed well on a vortex-mixer. After 30 min, absorbance was measured at 595 nm. For control Milli-Q water was used and same procedure was followed. The blank absorbance was subtracted from the absorbance of the sample before calculating the final concentration of monosaccharide (eg. glucose). It is important to note that the whole analytical procedure was carried out in the

dark because most of the reagents are light sensitive. The MCHO (free glucose) in growth medium was directly measured using the same carbohydrate procedure but without hydrolysis. The concentration of DPCHO is equal to the difference between TDCHO and MCHO (Pakulski and Benner, 1992).

$$[DPCHO] = [TDCHO] - [MCHO]$$

Calibration curves were prepared using glucose (0.5 to 3 µg /ml) as standard. Analytical variation of the method was ± 4%. The concentration of MCHO, DPCHO and TDCHO are expressed as glucose equivalent in µM C. The unit µM was obtained assuming that all monomers were hexoses. The unit µM C is obtained by multiplying µM values by 6 assuming 6 mol of carbon per mol of hexoses.

2.2.6.3 Determination of DURA

The concentrations of DURA in growth spent medium were determined according to Hung and Santschi (2001), which is based on the modification of the method of Filisetti-Cozzi and Carpita (1991) after pre-concentration by using cation exchange resin and freeze drying. The 10 ml of filtered spent growth medium were passed through cation exchange resin column (Dowex 50W-8X 50 to 200 mesh, H^+ form, Sigma Chemical Co.). The column was washed with three bed volumes of Milli-Q water to remove the adsorbed uronic acids from the cation exchange column. This washing procedure gave 90 to 96% recovery of uronic acid. Hung and Santschi (2001) reported 84 to 89% recovery of uronic acids using the same procedure. All the washings

(column passing solution) were combined, and reduced to a small volume using a rotary vacuum evaporator. To the 0.4 ml of the concentrated sample, 40 µl of 2 M sulfamic acid was added and the solution was stirred on a vortex mixer. Subsequently, 2.4 ml of 75 mM sodium tetraborate in concentrated sulfuric acid was added to the vial, and the vial was heated at 100 °C for 10 min in a boiling water bath. After cooling, 80 µl of 0.15% m-hydroxydiphenyl was added to the vial and mixed well by vortex mixer. The red color was developed. The absorbance was measured after 10 min, at 525 nm. Absorbance of Milli-Q water blank was subtracted from sample absorbance. Glucuronic acid was used as a standard compound for calibration. Concentration was expressed as glucuronic acid equivalent. The unit used is µM of glucuronic acid. The unit µM C is obtained by multiplying µM values by 6 assuming 6 mol of carbon per mol of hexoses. The coefficient of variation of the method was < 6%.

2.2.6.4 Monosaccharide composition of EPS

In order to find out monosaccharide composition, EPS was hydrolyzed with 2 N HCl for 3 h at 100 °C in ampoules flushed with nitrogen gas before sealing. After cooling, an internal standard (inositol) was added. Then sample was evaporated to dryness under reduced pressure at 40 °C for the removal of acid. The hydrolysate was dissolved in Milli-Q water and passed through a Dowex 50 W-X8 (H^+ form, 100 mesh; Sigma Chemical Co.) column using water as an eluent to isolate neutral sugars. The solution containing neutral sugars were concentrated to small volume (1 ml) using rotary evaporator. The

pH of the resulting solution was adjusted to 8-9 by adding 10% triethylamine solution in water for the hydrolysis of lactones. After about 30 min an excess $NaBH_4$ was added and kept 4-5 h in dark at room temperature to reduce the monosaccharides to the corresponding alditols. Excess $NaBH_4$ was decomposed by addition of glacial acetic acid. After the effervescence stopped, the solution was evaporated to dryness under reduced pressure. Boric acid was removed by repeated addition of methanol and evaporation to dryness. The samples were desiccated over KOH in vacuum to remove traces of water. The acetylation was performed by addition of 0.3 ml pyridine and 0.3 ml acetic anhydride (1:1) mixture and incubating the sample overnight at room temperature. The acetylating reagent was evaporated under reduced pressure and the resulting sample was desiccated overnight over KOH in vacuum. The residue was dissolved in Milli-Q water (4 ml) and the solution was extracted 3 times with equal amount of dichloromethane. The combined dichloromethane extracts were dried on anhydrous sodium sulphate and concentrated to 100 µl using the rotary vacuum evaporator and analyzed by capillary gas chromatography (GC)

A capillary gas chromatograph (Shimadzu, model-2010) equipped with a fused silica capillary column coated with CP Sil-88 (25 m, i.d. = 0.32 mm, df = 0.2 µm, VARIAN) and flame ionization detector (FID) was used to separate the alditol acetate mixture. Both detector and injector were maintained at 300 °C. The oven temperature was programmed as follows: 70 °C to 150 °C at 10 °C/min and then at 3 °C/min to 230 °C/min, at which it was maintained for 10 min.

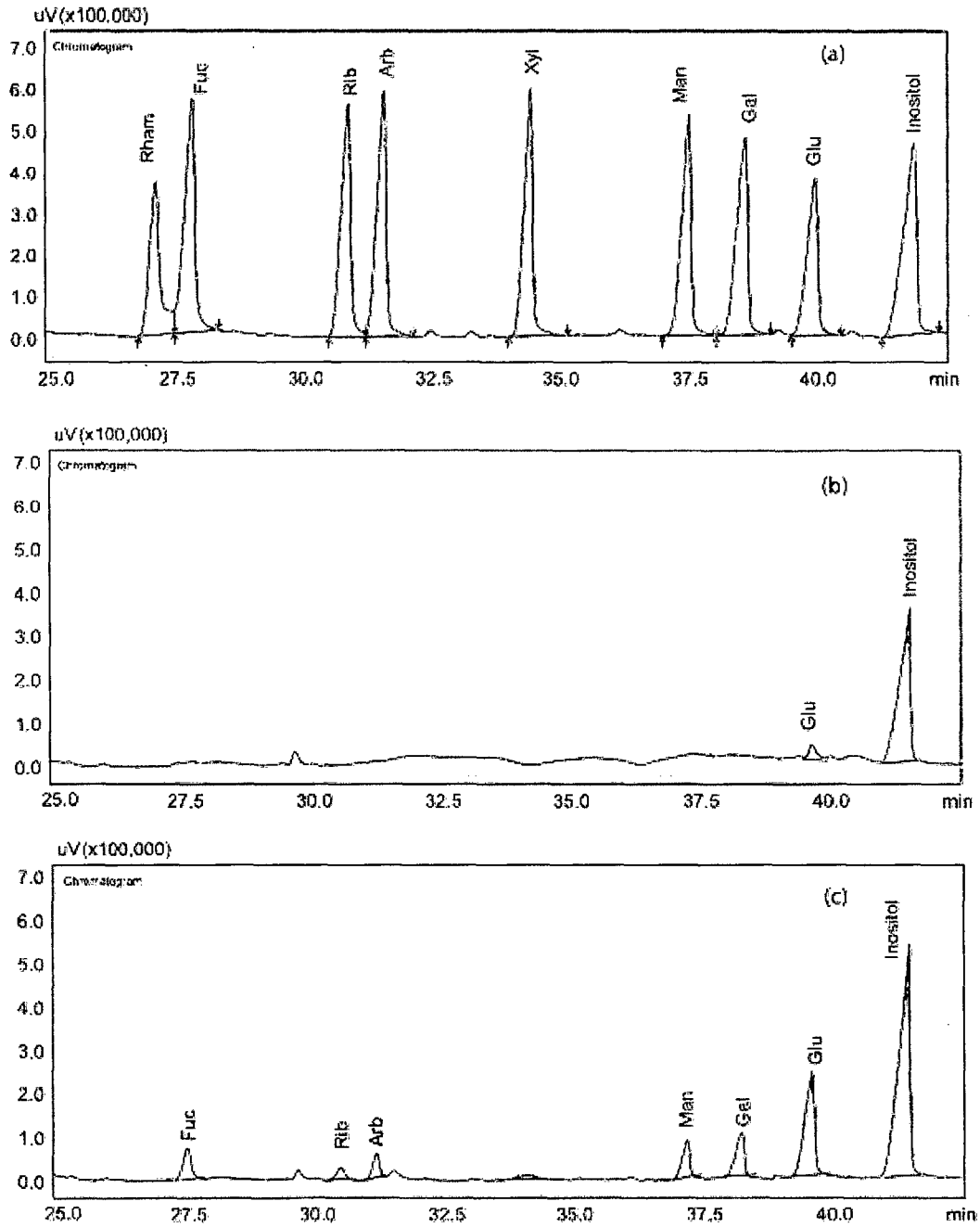


Fig. 2.1 Gas chromatographic trace of sugar alditol acetates of a standard mixture (a), blank (b), and EPS sample obtained from *Bacillus licheniformis* (c). Rham = rhamnose, Fuc = fucose, Rib = ribose, Arb = arabinose, Xyl = xylose, Man = Mannose, Gal = galactose, Glu = glucose and Inositol (internal standard).

Identification of the monosaccharide was done by comparing the retention time of unknown monosaccharide with that of standard monosaccharide (Fig. 2.1). Quantification was done using the data handling system (GC solution, software) installed in the instrument. In this study, total particulate neutral carbohydrate (TNPCHO) has been defined as the sum of all the identified monosaccharides. The contribution of individual monosaccharide to the TNPCHO is expressed as mol%. Analytical reproducibility of the GC method was ± 5.7%. Blank samples were treated and analyzed using same procedure. Blank values were negligible when compared to the samples. Blank values were subtracted from the sample values.

Uronic acids in the EPS:

A known amount of EPS sample was placed in a test tube and the uronic acids content was estimated as described above. Blank was run in the same way as for the sample except that 80 µl 0.15 % *m*-hydroxydiphenyl was replaced by 80 µl 0.5 % NaOH. For each sample a separate blank was used.

2.3 Results

2.3.1 Identification of bacteria

Genus assignment of the isolates was based on characteristics such as gram reaction, cellular morphology, physiological and biochemical tests (Table 2.1). All four cultures were Gram-positive, straight rods, spore forming, and produced creamy white circular colonies with smooth shiny surface when

grown on Zobell-Marine agar, and the culture were citrate and catalase positive.

Table 2.1. Biochemical and physiological characteristics and tentative identifications of four marine bacterial cultures.

Tests	Cultures			
	SS-14	Ti-28	CE-2	Bac
Gram stain	Gm +ve	Gm +ve	Gm +ve	Gm +ve
Cell morphology	Short rods	Short rods	Long rods, capsule around the cells	Long rods
Spore formation	+ve	+ve	+ve	+ve
Motility	+ve	+ve	-ve	-ve
Pigmentation	Creamy white	Creamy white	Creamy white	Creamy white
Colony shape	Small round	Small round	Big round	Big round
Oxidase	+ve	-ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve
Gelatin hydrolysis	-ve	-ve	+ve	+ve
Starch hydrolysis	-ve	+ve	+ve	+ve
Citrate	+ve	+ve	+ve	+ve
Nitrate reduction	-ve	-ve	+ve	+ve
Hugh-Liefson's medium	+ve	+ve	-ve	+ve
Acid from glucose	+ve	+ve	+ve	+ve
Acid from fructose	+ve	+ve	+ve	+ve
Acid from sucrose	+ve	+ve	+ve	+ve
Acid from lactose	-ve	-ve	-ve	-ve
Glucose assimilation	+ve	+ve	+ve	+ve
Fructose assimilation	+ve	+ve	+ve	+ve
Sucrose assimilation	+ve	+ve	+ve	+ve
Lactose assimilation	-ve	-ve	-ve	-ve
Tentative identification	***Bacillus pumilus***	***Bacillus* sp.**	***Bacillus subtilis***	***Bacillus licheniformis***

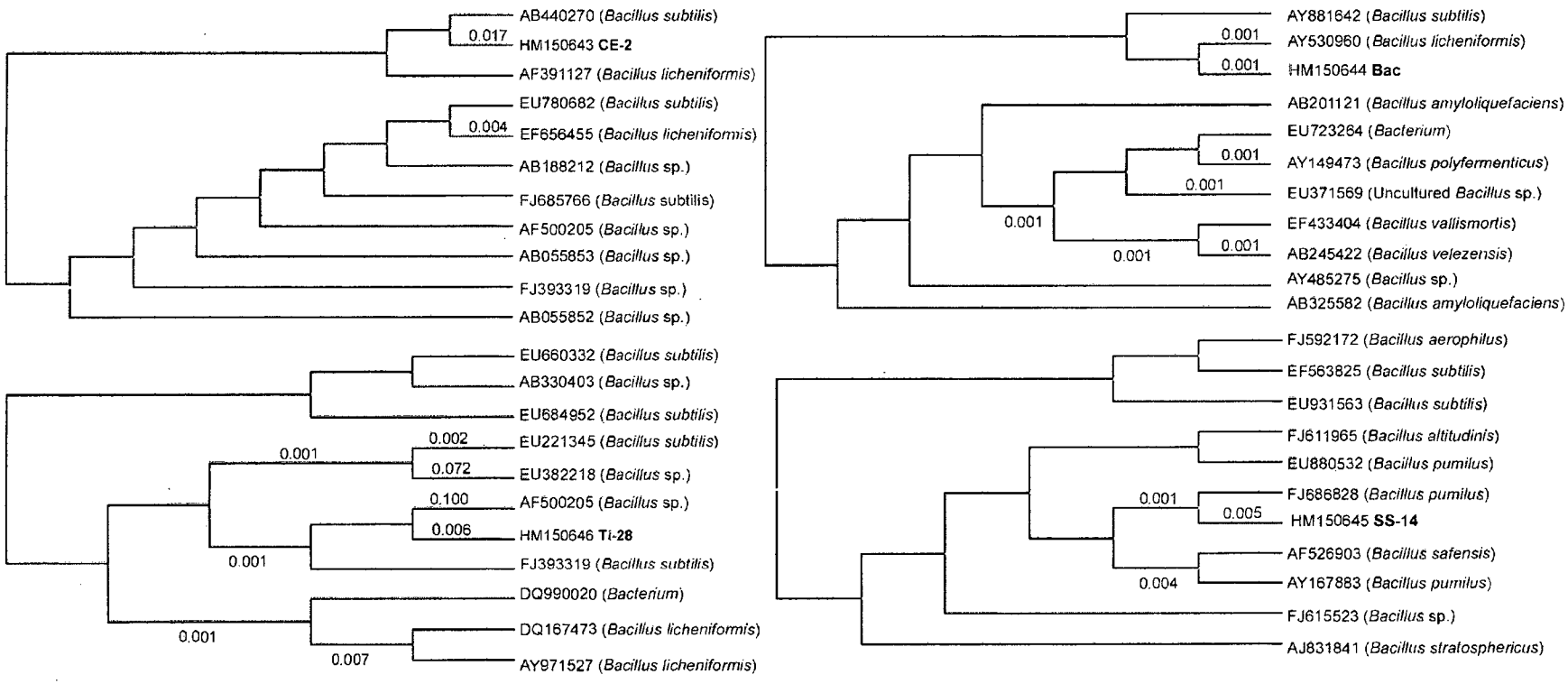


Fig. 2.2 Phylogenetic tree showing the relationships of the microbial culture based on 16S rDNA sequences. The tree was constructed using MEGA 3.1 software using neighbour joining method.

CE-2 and Bac cultures were non-motile, hydrolyzed gelatin and reduced nitrate to nitrite (Table 2.1). CE-2 cells have Cell capsules. The cultures SS-14 and Ti-28 were motile, and were negative for gelatin hydrolysis and nitrate reductase reaction (Table 2.1). These morphological, biochemical and physiological characteristics of cultures were similar to those described for *Bacillus* sp. in the Bergey's Manual of Systematic Bacteriology (1984) and thus the cultures were tentatively identified as *Bacillus* sp.

Further, cultures were identified as members of the genus *Bacillus subtilis* (CE-2), *Bacillus* sp. (Ti-28), *Bacillus licheniformis* (Bac) and *Bacillus pumilus* (SS-14) based on the 16S rDNA sequences (Fig. 2.2). Bacterial cultures CE-2, Ti-28, Bac and SS-14 were closely related to *Bacillus subtilis*, *Bacillus* sp., *Bacillus licheniformis* and *Bacillus pumilus*, respectively (Fig. 2.2), with similarity value ranging from 97 to 99%.

2.3.2 Growth and production of TDCHO, DPCHO and DURA by Bacillus spp.

To study the production of carbohydrates, growth curve experiment was performed for each *Bacillus* sp. wherein dissolved carbohydrates and growth (as optical density) were measured over the period of incubation (Fig. 2.3 and 2.4). All four cultures showed a lag phase of about 4 h. Maximal growth was achived at 20 h, 16 h, 14 h and 20 h, for *Bacillus subtilis*, *Bacillus* sp. *Bacillus licheniformis* and *Bacillus pumilus,* respectively (Fig 2.3a). The growth of *Bacillus* sp. and *Bacillus licheniformis* decreased after 14 to 16 h following the inoculation. *Bacillus pumilus* biomass was relatively higher than *Bacillus*

subtilis, Bacillus sp. and *Bacillus licheniformis*, especially after 24 h following the inoculation (Fig. 2.3a).

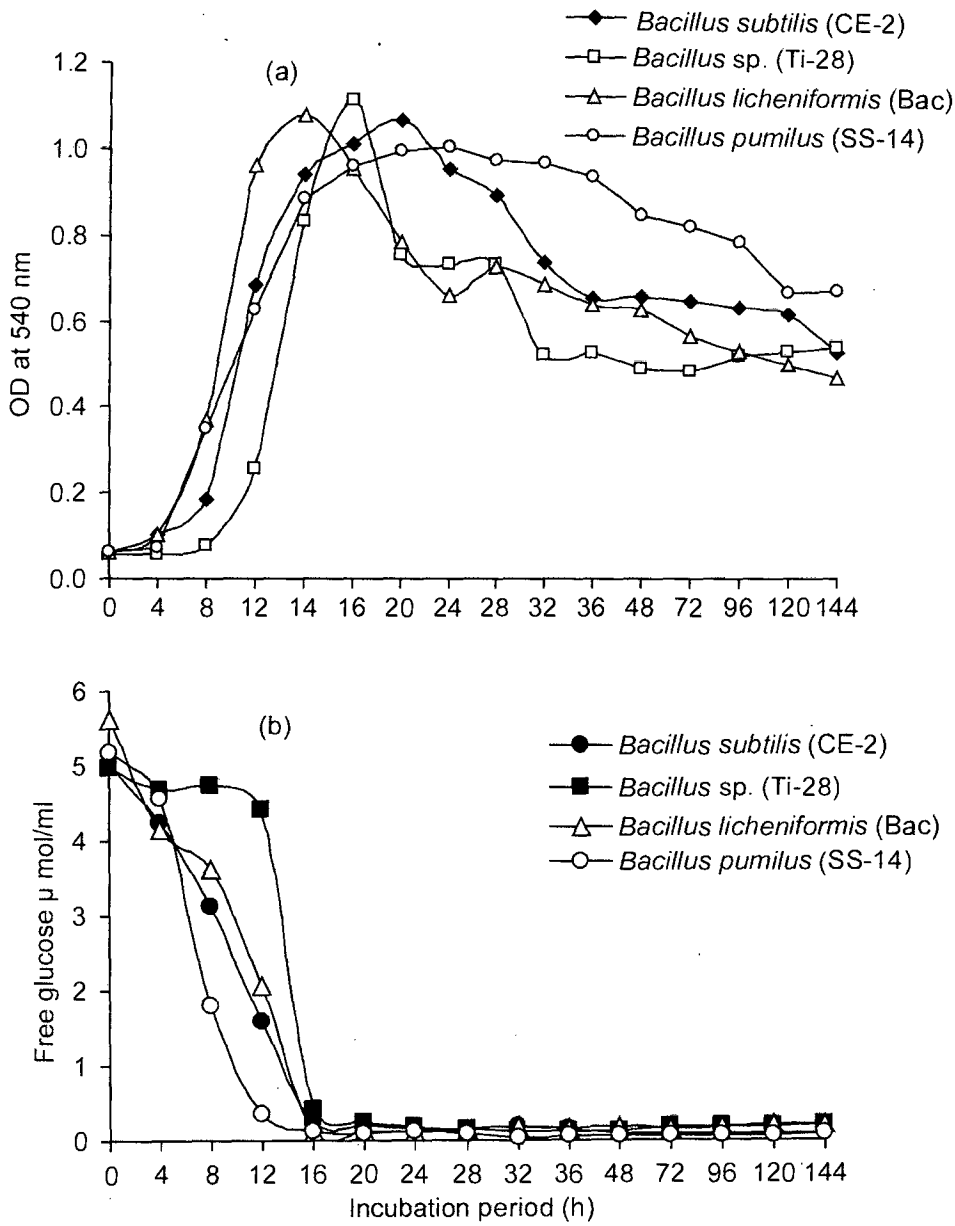


Fig. 2.3 Growth curve of *Bacillus subtilis* (CE-2), *Bacillus sp.* (Ti-28), *Bacillus licheniformis* (Bac) and *Bacillus pumilus* (SS-14) (a) and changes in substrate (glucose) concentrations during the period of incubation (b).

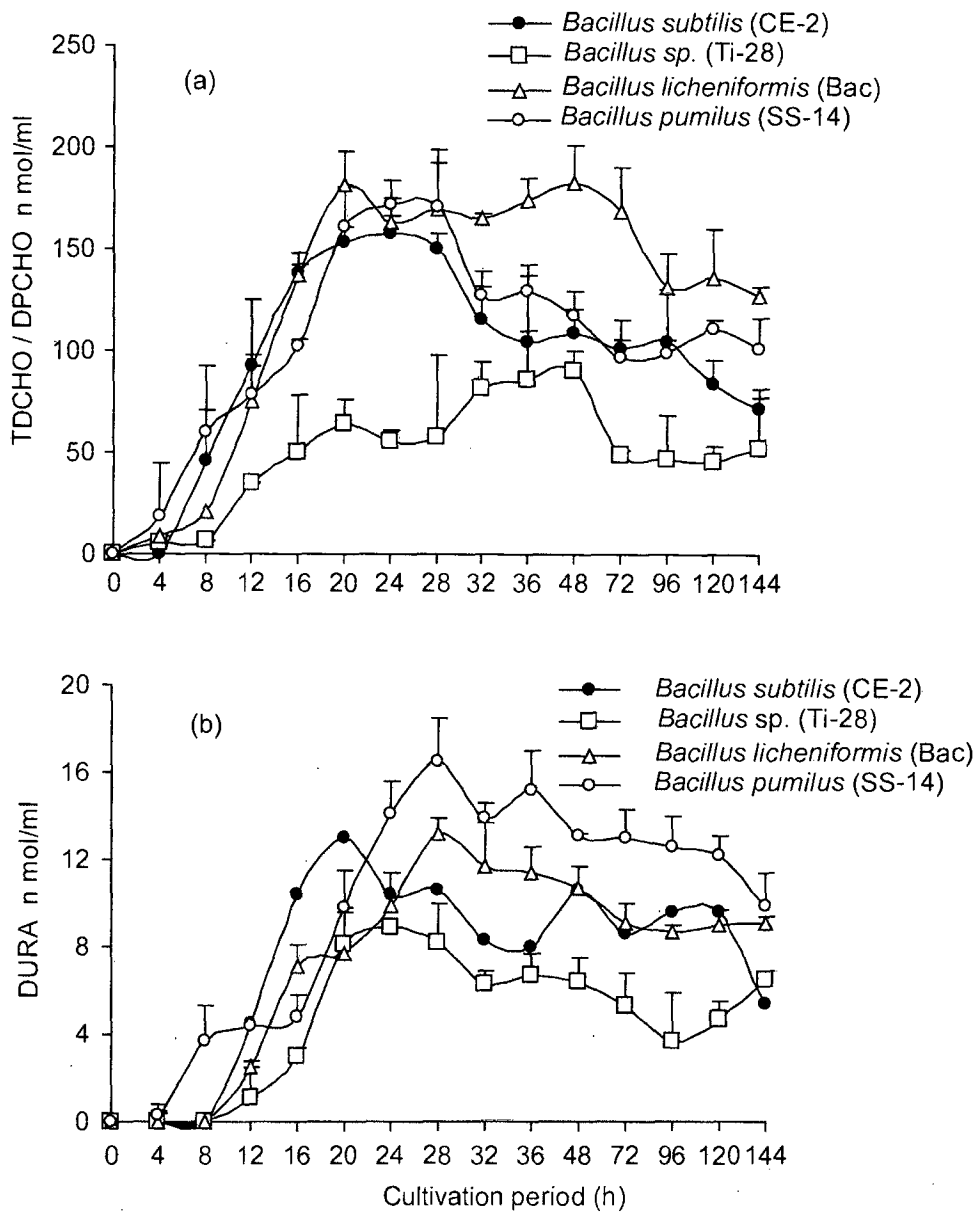


Fig. 2.4. Production of dissolved carbohydrates (TDCHO) or dissolved polysaccharide (DPCHO) (a) and dissolved uronic acids (DURA) (b) by the *Bacillus* cultures over the period of incubation.

Free glucose (MCHO) concentration decreased with the increase in bacterial growth (Fig 2.3, b). During the initial 16h of growth of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus,* glucose concentrations decreased

drastically. Thereafter, TDCHO increased as the cultures started producing the polysaccharides (Fig. 2.4a).

Production of DPCHO also increased with increase in bacterial growth (Fig. 2.4a). *Bacillus* spp. showed significant differences (one Way ANOVA, $p< 0.001$) in the production of TDCHO and DPCHO. DPCHO production by *Bacillus subtilis* (CE-2) (158 nmol/ml) and *Bacillus pumilus* (SS-14) (171 nmol/ml) was maximal at 24 h, following the inoculation. Whereas, for *Bacillus* sp. (Ti-28) and *Bacillus licheniformis* (Bac) maximal DPCHO production (90 nmol/ml and 182 nmol/ml) was noted at 48 h following the inoculation (Fig 2.4a). The production of DPCHO by *Bacillus licheniformis* was higher than *Bacillus* sp. (Fig. 2.4a).

DURA is acidic carbohydrate. Production of DURA was significantly different (one Way ANOVA, $p< 0.001$) for the four *Bacillus* spp. (Fig. 2.4b). For *Bacillus* sp. DURA production was very low during first 8 h following the inoculation (Fig. 2.4b). Thereafter, the concentration of the DURA increased as the period of incubation increased. DURA production by *Bacillus* sp. was higher (16.5 nmol/ml) than by *Bacillus pumilus* (8.9 nmol/ml) (Fig. 2.4b).

2.3.4 Chemical characterization of EPS

EPS is composed of organic carbon, nitrogen, neutral carbohydrates and uronic acids (Table 2.2). Organic carbon and nitrogen content of the EPS varied from 15.33 to 28.08 μmol C mg^{-1} and 0.93 to 2.51 μmol N mg^{-1} of EPS, respectively (Table 2.2). C/N ratio of EPS showed large variations (8.72 to 19.74). (Table 2.2). Neutral sugars concentration was higher (6.49 μmol C

mg^{-1}) in *Bacillus* sp. and lower (0.76 µmol C mg^{-1}) in *Bacillus licheniformis* (Bac) (Table 2.2). Higher neutral sugar concentrations were associated with higher C/N ratio. EPS produced by *Bacillus subtilis* has high content of uronic acids (0.70 µmol C mg^{-1}) as compared to *Bacillus sp.* (0.49 µmol C mg^{-1}), *Bacillus licheniformis* (0.46 µmol C mg^{-1}) and *Bacillus pumilus* (0.41 µmol C mg^{-1}), respectively (Table 2.2). Capillary gas chromatographic analysis showed large differences in monosaccharide composition (Table 2.2).

Table 2.2 Chemical and monosaccharide composition of iso-propanol precipitated EPS produced by marine *Bacillus* cultures.

Bacteria	*Bacillus subtilis* (CE-2)	*Bacillus* sp. (Ti-28)	*Bacillus licheniformis* (Bac)	*Bacillus pumilus* (SS-14)
Chemical composition of EPS				
Organic carbon µmol C mg^{-1}	28.08	18.33	21.17	15.33
Total nitrogen µmol N mg^{-1}	2.50	0.93	2.43	1.21
C/N ratio	11.23	19.74	8.72	12.63
Neutral sugars µmol C mg^{-1}	6.10	6.49	0.76	3.04
Uronic acids µmol C mg^{-1}	0.70	0.49	0.46	0.41
Monosaccharides (Mol %)				
Rhamnose	-	-	-	-
Fucose	-	-	16.27	-
Ribose	-	-	1.62	-
Arabinose	1.49	-	7.29	-
Xylose	1.06	-	-	16.28
Mannose	-	0.26	3.65	15.08
Galactose	33.26	5.22	4.45	30.25
Glucose	64.19	94.53	66.71	38.42

- = not detected

Glucose, galactose and mannose were the major component of the EPS. Fucose (16.27 mol%), arabinose (7.29 mol%) and ribose (1.62 mol%) were relatively more abundant in the EPS produced by *Bacillus licheniformis*. The contribution of glucose to total sugar (mol%) was 64.19% in *Bacillus subtilis*, 94.53% in *Bacillus sp.*, 66.71% in *Bacillus licheniformis* and 38.42% in *Bacillus pumilus* (Table 2.2).

2.4 Discussion

2.4.1 Bacterial growth and dissolved carbohydrate production by Bacillus sp.

Four marine *Bacillus* spp. were selected to assess their potential to produce dissolved carbohydrates or dissolved polysaccharides (EPS) because of their abundance in seawater and coastal sediments of the west coast of India (Parvathi et al., 2009). *Bacillus* spp. are involved in biofilm formation (D'souza, 2004), dissolved organic matter production (Ogawa et al., 2001) and thus may play an important role in biogeochemical and ecological processes in aquatic environments. Based on the morphological, biochemical and physiological characteristics, all the four cultures were tentatively identified up to generic level as *Bacillus* sp. (Table 2.1). Based on 16S rDNA molecular technique, these cultures were identified as *Bacillus subtilis* (CE-2), *Bacillus sp.* (Ti-28), *Bacillus licheniformis* (Bac) and *Bacillus pumilus* (SS-14) (Fig. 2.2).

Energy requirement for the biosynthesis of cell constituents depend on the availability of a suitable supply of low molecular weight compounds (eg.

glucose). These compounds serve as precursors or raw materials for the production of carbohydrates including EPS (Stoderegger and Herndl, 1998). Glucose was utilized by bacteria as source of carbon and energy (Fig. 2.3). This was evident from the decrease in glucose concentration and increase in cell density (Fig. 2.3). Bacteria grew in batch cultures, and up to 59% of glucose was converted to dissolve polysaccharides. Similarly, Stoderegger and Herndl (1998) reported that up to 45% of organic carbon like glucose incorporated by bacteria is converted into EPS, which is then released into the surrounding waters.

Bacteria are known to produce gel-like secretions called exopolymers. Concentrations of polysaccharide produced by *Bacillus subtilis*, *Bacillus* sp. *Bacillus licheniformis* and *Bacillus pumilus* increased with growth and maximum production was observed during the stationary phase of growth. Similar observations were reported for other EPS producing bacterial cultures (Majumdar et al., 1999; Jain et al., 2007). Polysaccharides production by *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilis* was relatively greater than that observed with Bacillus sp. This suggests that extracellular release by bacteria is influenced by species (Decho, 1990). The production of polysaccharides by microorganisms is influenced by various factors including the physiological state of the organism, age of the organism, nutrient levels and composition (Decho, 1990, Lee et al., 2007). For example, highest EPS production (1.30 μmol ml^{-1}) was recorded when *P. aeruginosa* cells were grown in nitrogen free medium (Borgio et al., 2009).

2.4.2 DURA production by Bacillus sp.

To the best of our knowledge, this is the first report on the production of DURA by marine bacteria. Acidic carbohydrates such as uronic acids are produced by bacteria in response to low nutrient and high concentrations of metals (Costerton 1984; Leppard 1993). They are surface-active compounds and involved in the removal of toxic metal ions, detoxification of toxic chemicals, adsorption of dissolved organic matter (Decho, 1990) and microbial adhesion and biofilm development (Decho, 1990; Garg et al., 2009; Jain and Bhosle, 2009). DURA concentrations were significantly different (One way ANOVA, $p< 0.05$) between the four *Bacillus* spp. and its production increased with growth of bacteria and higher uronic acid is produced during stationary growth phase (Fig. 2.4b). Bacteria are important contributors to uronic acids in marine environment. For example, uronic acids concentration showed significant positive relationships with total culturable bacterial counts in the Bay of Bengal (Khodse et al., 2007). Furthermore, production of uronic acids by bacteria is also influenced by nutrient concentrations in the growth medium or in natural waters (Leppard, 1993). Khodse et al. (2010) also observed that, DURA concentrations showed inverse correlation with the concentrations of silicates and nitrate during pre-monsoon season in the Mandovi estuary. In view of this, nutrient depletion may be the reason for higher production of DURA during the stationary growth phase. In aquatic environment, uronic acids play an important role in biofilm formation, aggregate formation, formation of flocs (Leppard, 1997) and altering the surface characteristics of suspended particles and modifying the solubility of

suspended molecules (Leppard, 1997; Santschi et al., 1998). The observed variations in the production of DURA may influence the above processes in marine environment.

2.4.3 Chemical characterization of EPS produced by Bacillus cultures

EPS derived from various sources behave differently due to the dissimilarity in their chemistry (Hoagland et al., 1993). In the present study, EPS derived from four *Bacillus* cultures showed considerable differences in their chemistry. Bacterial EPS had a high amount of carbon and low amount of nitrogen and a high C/N ratio (Table 2.2). EPSs from different strains may be different in yield, chemical composition, structure and physical properties, but may have some common characteristics. Most EPSs produced by marine bacteria are heteropolysaccharides consisting of three or four different monosaccharides (Decho, 1990). The monosaccharides may be pentoses, hexoses, amino sugars or uronic acids. EPS differed in their chemical composition, as shown by colorimetric and gas chromatographic analysis (Table 2.2). EPS produced by *Bacillus* sp. contain glucose, galactose, mannose, xylose, arabinose, ribose, fucose and uronic acids. The presence of these sugars suggests that the EPS is a heteropolysaccharide in nature. Glucose was the major sugar component (37 to 94 mol %) followed by galactose, mannose, xylose, arabinose, ribose, and fucose. The EPS produced by each *Bacillus* sp. is chemically different, it suggest that the composition of the EPS differs within bacterial species. Moreover, growth phase, and nutrient status may influence the quality and composition of EPS produced by marine bacteria (Decho,

1990). For example, Beech et al. (2000) reported that EPS produced by *Pseudomonas* sp. was chemically different in planktonic and biofilm growth conditions. EPSs from marine bacteria usually possess hydroxyl and carboxyl groups, which confer a net negative charge and acidic properties to the EPSs. Up to 20–50 % of these EPSs may be accounted as uronic acids (Decho, 1990). Uronic acid content in the EPS produced by *Bacillus subtilis* was two times higher than *Bacillus* sp., *Bacillus licheniformis* and *Bacillus pumilus*.

Uronic acids are present in all the bacterial EPS indicating that EPS was acidic in nature. Kennedy and Sutherland (1987) surveyed a wide range of both marine and fresh water bacteria. They found that while the specific composition differed between isolates and all isolates contains uronic acids and several neutral polysaccharides, some of the marine strains having high acetyl content. Humphrey et al. (1979) characterized the EPS of the gliding bacterium (*Flexibacter* BH3), and found it to be glycoprotein containing glucose, galactose, fucose, and some uronic acid. The composition of EPS may also vary according to the growth phase of bacteria (Christensen et al., 1985). Moreover, culture conditions generally do not affect the types of monosaccharides in an EPS, they do affect the functional properties of the polysaccharide such as molecular weight, and monosaccharide ratios (Arieas et al., 2003).

2.5 Conclusions

The present study demonstrate that *Bacillus subtilis* (CE-2), *Bacillus licheniformis* (Bac) and *Bacillus pumilus* (SS-14) are effective producers of exopolysaccharides, which are rich in uronic acids. Polysaccharide production is higher when the cultures reach the stationary phase. EPS produced by *Bacillus subtilis* (CE-2), *Bacillus licheniformis* (Bac) and *Bacillus pumilus* (SS-14) were chemically different heteropolysaccharides and were acidic in nature.

Chapter 3A

Production of dissolved carbohydrates and uronic acids by marine diatom *Amphora rostrata Wm.Sm.*

3A.1 Introduction

In aquatic environments, diatoms are the most diverse group of eukaryotic organisms. They are found in both freshwater and marine environments as well as on wet surfaces (Medlin, 2002; Underwood and Paterson, 1993). They are adapted to two different living conditions, either as free floating (planktonic) or attached (benthic) (Alekseeva et al., 2005). They are important primary producers; and CO2 fixed by diatoms in the oceans may count up to ~40% of total primary production (Mann, 1999). Fixed carbon is used as structural carbohydrates or stored in the form of chrysolaminaran or secreted outside the cells as extracellular polymeric substance (EPS) (Hoagland et al., 1993). Diatoms secretes 20-80% of the fixed carbon as EPS (Goto et al., 2001). In addition, proteins (Staats et al., 1999), glycoproteins (Chiovitti et al., 2003), uronic acids (Chiovitti et al., 2003; de Brouwer and Stal, 2002; Staats et al., 1999; Wustman et al., 1997) together contribute a small fraction of the secreted EPS.

Carbohydrate production by diatoms has been demonstrated in numerous studies done under laboratory conditions. Such studies indicate that type, quality and composition of carbohydrate are influenced by species (Myklestad, 1974; Leandro et al., 2003), nutrient status, growth phase (Urbani et al., 2005), and pH of culture medium (Thornton, 2009). Claquin et al. (2008) reported that transparent exopolymeric particles (TEP) production is significantly affected by temperature and diatom species. Diatoms produce water soluble small molecular weight polysaccharides that are released into the surrounding water (de Brouwer and Stal, 2002). Diatoms produce

heteropolysaccharides and acidic polysaccharides known as uronic acids. These polymers influence ecological and/or biological functions in aquatic environments. Polysaccharide protects silicious cell walls from herbivores, holds pregametangial cells together and raises the cells from the surface of substrata to avoid competition for light and nutrients (Hoagland et al., 1993). Polysaccharide mainly serves as food for other organisms like bacteria (Giroldo et al., 2003). It holds the sediment particles together which results in lower erosion (de Brouwer et al., 2005; Stal, 2003; Yallop et al., 2000). Diatoms are important components of biofilms which are known to reduce performance of ships by increasing fuel consumption (Chiovitti et al., 2003).

In recent years, there is growing interest in carbohydrate production studies by diatom because they play important role in global carbon cycling and influencing the dynamics of microorganisms in marine environments. In this chapter, our aims were 1) to study growth and carbohydrate production by *Amphora rostrata*, a marine fouling diatom and 2) to characterize the polysaccharides produced by the diatom.

3A. 2 Materials and Methods:

3A.2.1 Culture conditions and source of algal material

Amphora rostrata Wm. Sm., a pennate diatom was used for this study. The culture was maintained at the Marine Corrosion and Materials Research Division, National Institute of Oceanography, Goa, India. *A. rostrata* was originally isolated from biofilms developed on a glass slide immersed in the marine waters of Dona Paula Bay, west coast of India (15°.31'N, 73°.59'E)

(Bhosle et al., 1995). A stock culture was routinely maintained at room temperature (28 °C ±2 °C) in the laboratory by weekly re-inoculation into fresh f/2 medium (Appendix II), prepared in autoclaved seawater (~35 psu) enriched with nutrients (Guillard and Ryther, 1962). Cells were grown on a shaker (30 rpm) in Erlenmeyer flasks (100 ml) containing 10 ml of culture medium and were maintained at 28 ±2 °C under a light photocycle of 12h:12h light:dark (L:D) condition. Illumination was provided by two 40 W white fluorescent tubes.

3A.2.2 Preparation of axenic algal culture

Before use, the diatom cultures were made bacteria free by treating with penicillin G (1000 mg L^{-1}), streptomycin sulphate (500 mg $L^{-1)}$ and chloramphenicol (100 mg L^{-1}) for 24 h and repeating the treatment for another 24 h. The culture was then transferred to antibiotic-free medium (f/2) and cultivated as above.

3A.2.3 Growth and carbohydrate and uronic acid production by A. rostrata

Cells were grown in Erlenmeyer flasks (1 liter) under a light photo-cycle of 12:12 L:D condition as above to assess the growth and the production of dissolved carbohydrate and uronic acids. Ten experimental flasks (1 liter) each containing 200 ml of f/2 medium were inoculated with 20 ml of 5 day old culture (1.8×10^5 cells ml^{-1}) of the *A. rostrata*. Flasks were incubated at room temperature (28 ±2 °C) as above. One flask was removed immediately after

inoculation and then every day over the 10 d period of cultivation. After removal of the flask, a known volume (10-15 ml) was filtered through pre-combusted (400 °C for 4 h) GF/F filter (0.7 µm pore size, Whatman) for chlorophyll *a* (Chl *a*) analysis and the filtrate was used for nutrient analysis. A small volume (1 ml) of growth medium was fixed with Lugol's iodine and stored at 4 °C for analysis of cell number. Further, filtrate was passed through polycarbonate filter (0.2 µm pore size, Nucleopore) and used for the analysis of dissolved carbohydrates and uronic acids.

3A.2.4 Analytical methods

The cells retained on GF/F filter was treated with 90% acetone in darkness for 24 h at 4 °C to extract Chl *a*. Samples were centrifuged (3000 g for 10 min) and absorbance of the supernatant was measured at 665 nm and 750 nm in a UV-Vis spectro-photometer (Shimadzu Model UV-1700) (Parsons et al., 1984). Cell numbers were counted using haemocytometer and Nikon microscope (magnification 100X). The nutrients, nitrate, phosphate, and silicate were determined following the procedure described by Parsons et al. (1984).

For the dissolved organic carbon (DOC) 0.5 ml of 0.22 µm filtered spent medium was transferred in test tube and dried in an oven at 50 °C. Thereafter, DOC concentration was analyzed following the method of Parsons et al. (1984). Free monosaccharides (MCHO) and total dissolved carbohydrates (TDCHO) were analyzed following method described in chapter 2. The concentrations of dissolved uronic acids (DURA) were

measured by the method of Filisetti-Cozzi and Carpita (1991) as described in chapter 2. Monosaccharide composition of TDCHO was determined by gas chromatographic method (D'souza et al., 2005). Ten milliliter of 0.22 µm filtered spent growth medium was hydrolyzed with 2 N HCl for 3 h at 100 °C. The released neutral sugars were determined following the method described in chapter 2.

3A.3. Results

3A.3.1. Changes in Chl a, cell count and nutrient concentration

The concentration of Chl *a* and cell count increased over the period of cultivation and reached maximal on d 7 and d 8 following the inoculation, respectively (Fig. 3A.1). Thereafter, a small decrease was observed for both the Chl *a* and cell count. Nitrate, phosphate and silicate concentration decreased rapidly during the first 6 d following inoculation (Fig. 3A.1b).

3A.3.2 Production of DOC, TDCHO, MCHO and DPCHO by A. rostrata

DOC concentration increased from 49 µM C at d 0 to 500 µM C at d 10 of the cultivation period (Fig. 3A.2). Maximal DOC concentration was recorded at d 10 of the cultivation period (Fig. 3A.2). TDCHO and DPCHO concentration increased over the period of cultivation and reached a maximal value (26.87 µM and 21.16 µM) at d 9 following inoculation.

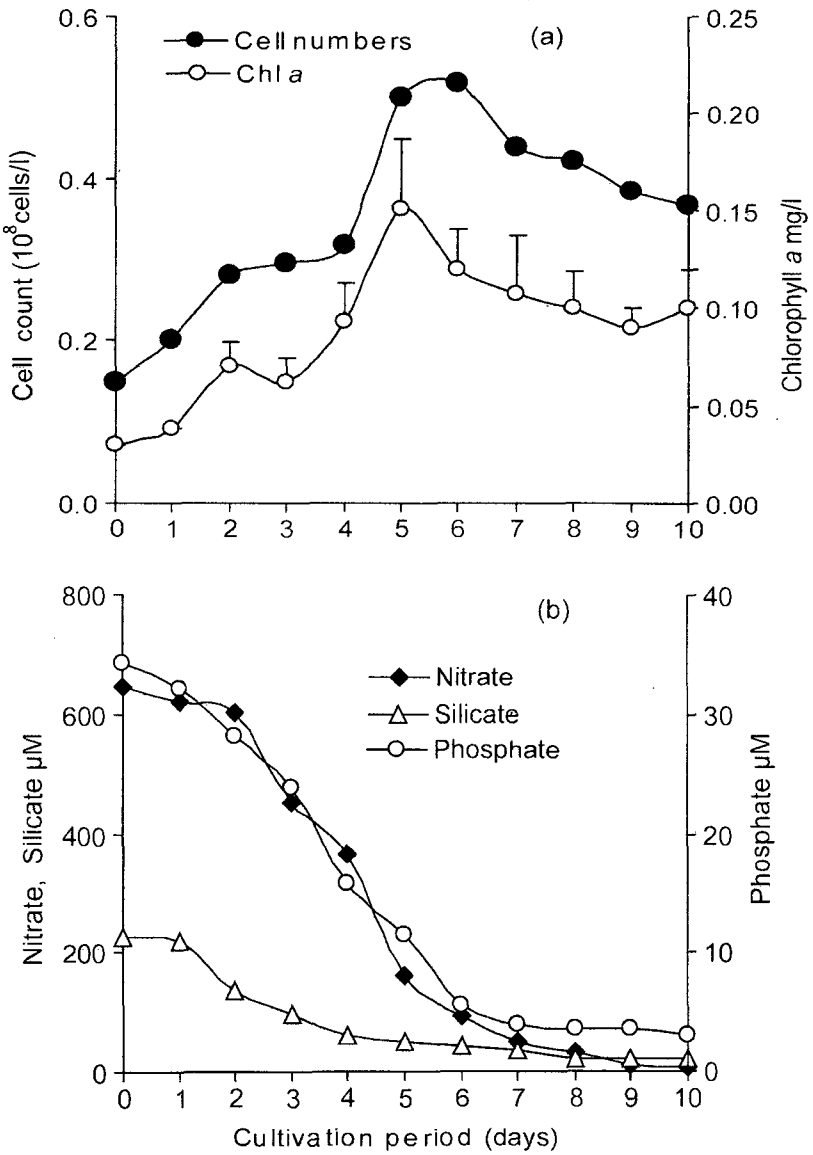


Fig. 3A.1. Growth of the diatom *Amphora rostrata* (measured as Chl *a* and cell count) (a) and changes in concentrations of nitrate, phosphate and silicate (b) during the cultivation period.

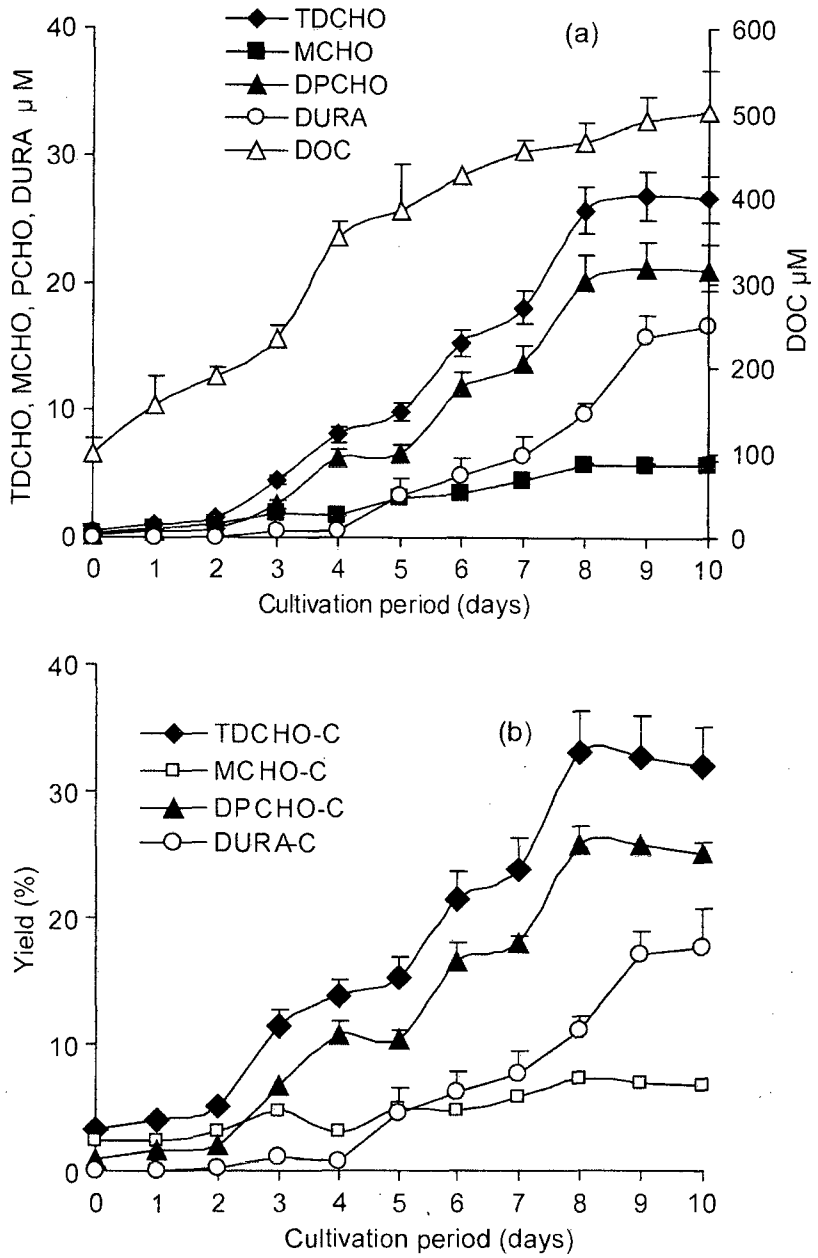


Fig. 3A.2. Production of dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), free monosaccharides (MCHO), dissolved polysaccharides (DPCHO), and dissolved uronic acids (DURA) by *Amphora rostrata* (a) and yields of carbohydrates (carbohydrate-C to DOC %)(b) during the cultivation period.

Thereafter, concentrations of DPCHO and TDCHO did not change much over the period of cultivation (Fig. 3A.2). MCHO concentrations increased and reached a maximal value of 5.71 μM at d 9 following the inoculation (Fig. 3A.2). In general dissolved carbohydrate carbon increased during growth of *A. rostrata* (Fig. 3A.2b). In the present study, TDCHO-C, MCHO-C and DPCHO-C accounted for 33%, 6% and 26% of DOC over the period of cultivation. DPCHO constituted dominant fraction (40 to 78%) of TDCHO pool.

DURA was not detected during initial two days following the inoculation. Thereafter DURA concentrations increased from 0.07 μM at d 3 to 16.55 μM at 10 d following inoculation (Fig. 3A.2a). DURA yield (DURA-C/DOC %) increased and reached maximal value (18%) at d 10 following the inoculation.

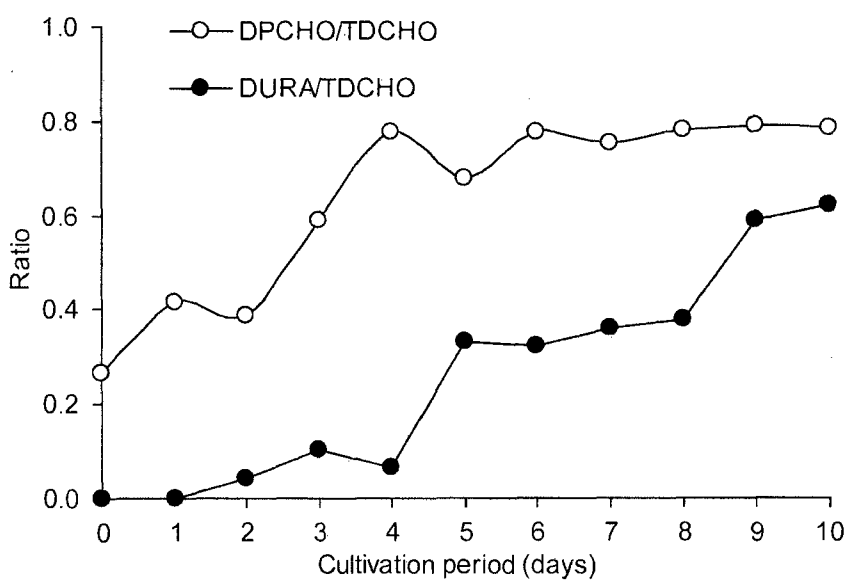


Fig. 3A.3 Changes in DPCHO/TDCHO (—○—) and DURA/TDCHO (—●—) ratios during the cultivation period.

Table 3A.1. Daily production rates (pg cell^{-1} d^{-1}) of dissolved extracellular carbohydrates by marine diatoms when grown under laboratory condition.

Organisms	Growth phase	Dissolved carbohydrate	Dissolved carbohydrate pg cell^{-1}d^{-1}	Light:Dark	References
Chaetoceros affinis	Exponential	DPCHO	42.0	-	Myklestad et al., 1989
Chaetoceros affinis	Stationary	DPCHO	20	-	Myklestad et al., 1989
Stauroneis amphionys	Log/stationary	DPCHO	2.4	-	McConville et al., 1999
Navicular perminuta	Log/stationary	DPCHO	28.4	14:10	Smith and Underwood, 2000
Nitzschia frustulum	Trasition	DPCHO	51.3	14:10	Smith and Underwood, 2000
Nitzschia frustulum	Trasition	DPCHO	21.2	14:10	Smith and Underwood, 2000
Nitzschia sigma	Trasition	DPCHO	42.2	14:10	Smith and Underwood, 2000
Nitzschia sigma	Trasition	DPCHO	6.3	14:10	Smith and Underwood, 2000
Surirella Ovata	Trasition	DPCHO	55.8	14:10	Smith and Underwood, 2000
Amphora exigua	logarithmic	DPCHO	4.4	-	Boulcott, 2001
Amphora exigua	logarithmic	DPCHO	8.7	-	Boulcott, 2001
N. cryptocephala	Log/stationary	DPCHO	2.6	-	Faraloni et al., 2003
N. gracilis	Log/stationary	DPCHO	2.5	-	Faraloni et al., 2003
Cylindrotheca closterium	Log/stationary	DPCHO	2.1	16:8	Urbani et al., 2005
Thalassiosira psedonana	Stationary	DPCHO	11.4	16:8	Urbani et al., 2005
S. costatum	Stationary	DPCHO	0.27	16:8	Urbani et al., 2005
Amphora rostrata	Exponential	TDCHO	2.24 ±1.62	12:12	This study
Amphora rostrata	Stationary		9.86 ±3.38	12:12	This study
Amphora rostrata	Exponential	MCHO	0.82 ±0.30	12:12	This study
Amphora rostrata	Stationary		2.17 ±0.67	12:12	This study
Amphora rostrata	Exponential	DPCHO	1.42 ±1.36	12:12	This study
Amphora rostrata	Stationary		7.69 ±2.72	12:12	This study
Amphora rostrata	Exponential	DURA	0.48 ±0.53	12:12	This study
Amphora rostrata	Stationary		5.18 ±3.07	12:12	This study

TDCHO = total dissolved carbohydrate; MCHO = free monosaccharides; DPCHO = polysaccharides; DURA = dissolved uronic acids.

Dissolved carbohydrate production varied with growth phase of *A. rostrata* (Table 3A.1). The production of TDCHO, MCHO, DPCHO and DURA was higher after 7 d of the cultivation period (Table 3A.1). TDCHO, DPCHO and DURA concentration showed significant inverse correlation with nitrate, phosphate and silicate during the growth period (Table 3A.2). DPCHO/TDCHO and DURA/TDCHO ratios changed during the growth of *A. rostrata* (Fig. 3A.3). DPCHO/TDCHO and DURA/TDCHO ratios increased with the cultivation period (Fig. 3A.3).

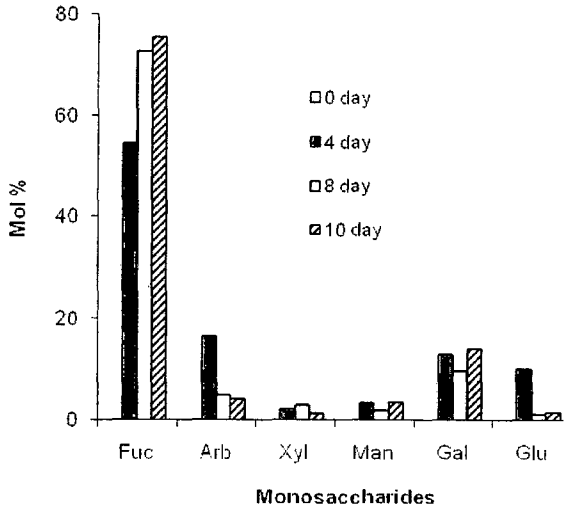
Table 3A.2. Correlation coefficients (r) for the relationships between nutrients and various dissolved carbohydrate species.

Parameters	TDCHO (r)	DPCHO (r)	DURA (r)
Nitrate	-0.935	-0.927	-0.821
Phosphate	-0.924	-0.917	-0.791
Silicate	-0.836	-0.824	-0.691

r = correlation coefficient

3A.3.3 *Monosaccharide composition of TDCHO*

Gas chromatography indicates the presence of fucose, arabinose, xylose, mannose, galactose and glucose in TDCHO (Fig. 3A.4). Differences in monosaccharide composition over the period of cultivation are shown in figure 3A.4. Fucose was major monosaccharide and increased from 55 to 75 (mol%) during the cultivation period whereas, glucose (mol%) concentration decreased (Fig. 3A.4).



3A.4. Monosaccharide composition (mol%) of TDCHO produced by *Amphora rostrata* over the period of cultivation.

3A.4. Discussion

3A.4.1 Growth and carbohydrates production by A. rostrata

A. rostrata was grown under 12:12 L:D conditions for 10 d. The growth of *A. rostrata* diatom was assessed by monitoring the Chl *a* concentration and cell count at one day intervals over the period of incubation. The concentration of Chl *a* and cell count increased over the period of cultivation and reached maximum on d 7 and d 8, respectively after the inoculation (Fig. 3A.1). Thereafter, small decrease was observed for the Chl *a* and cell count probably due to nutrient deficient condition in the growth medium. Production of DOC by *A. rostrata* increased over the period of cultivation. MCHO concentration also increased with the cultivation period and reached maximal at d 9, following inoculation. *A. rostrata* produced of TDCHO and DPCHO. These carbohydrates are involved in biofilm formation (Khandeparker and

Bhosle, 2001). Production of TDCHO and DPCHO increased with growth and maximal concentrations were recorded after 7 d period of cultivation. Many diatoms produce relatively large amounts of polysaccharides during the stationary growth phase (Myklestad et al., 1989; Smith and Underwood, 2000). For example, EPS production by *Navicula subinflata* Grun, a marine fouling diatom, was also highest during the stationary phase of growth (Bhosle et al., 1995). Concentrations of TDCHO and DPCHO produced by *A. rostrata* were significantly (One way ANOVA, p <0.001) higher in stationary phase than logarithmic growth phase (Table 3A.1).

Higher concentration of DPCHO was associated with lower concentration of nitrate, silicate and phosphate in the growth medium (Fig. 3A.1b). The relationship between nutrient concentrations and concentrations of TDCHO and DPCHO found in this work is in agreement with earlier studies conducted with other diatom species (Myklestad and Haug, 1972; Leandro et al., 2003; Urbani et al., 2005). P-limitation can also cause an increase in polysaccharides production by diatoms. Polysaccharide production by *C. closterium* cells was higher under P-limitation than N-limitation, possibly because of higher photosynthetic rate under P-limited conditions (Alcoverro et al.; 2000). In natural waters, TDCHO and DPCHO concentration influence bacterial adhesion. The concentrations of dissolved carbohydrates in Dona Paula Bay showed positive relationship with bacterial adhesion (Jain and Bhosle, 2009).

Cells normalised data showed slightly different pattern. Cell-normalised production rates for TDCHO ranged from 0.65-13.01 pg $cell^{-1}$ d^{-1} (average =

5.71 pg cell^{-1} d^{-1}),for MCHO from 0.47-2.78 pg cell^{-1} d^{-1} (average 1.44 pg cell^{-1} d^{-1}), for DPCHO from 0.17-10.27 pg cell^{-1} d^{-1} (average 4.27 pg cell^{-1} d^{-1}), and for DURA, from 0.0-8.75 pg cell^{-1} d^{-1} (average 2.5 pg cell^{-1} d^{-1}) (Table 3A.1). Cell normalised polysaccharide production by *A. rostrata* was within the range of values reported earlier for other diatom species (Table 3A.1). Boulcott (2001), reported that the three diatom taxa showed significantly higher rates of carbohydrate production per cell when in logarithmic growth phase than in stationary growth phase.

Carbohydrates yield is a useful indicator to assess the digenetic state of organic matter. For *A. rostrata* TDCHO-C, DPCHO-C and MCHO-C accounted for 33%, 26% and 7% of DOC, respectively. This indicates that TDCHO was the dominant fraction of DOC pool accounting for the major proportion of freshly produced DOC by *A. rostrata.* DPCHO accounted for 40 to 78 % of TDCHO during the cultivation period.

3A.4.2 DURA production by A. rostrata

Many diatoms produce dissolved acidic carbohydrate such as uronic acids. Uronic acids are important as surface active compounds due to their negative charge. In aquatic environments, uronic acids are important in several marine processes described in earlier chapter 2. Production of DURA increased over the cultivation period *of A. rostrata*. Higher production of DURA was observed in stationary growth phase when nutrient concentration was low in growth medium. Production of uronic acid is influenced by growth phase, levels of nutrient and metal ion concentrations in growth medium or in natural waters

(Bergamaschi et al., 1999; Hung et al., 2003). Uronic acids showed positive correlation with Chl *a* in the natural waters indicating that the phytoplankton was a source of uronic acids (Hung et al., 2003; Khodse et al., 2007). Bellinger et al. (2009) suggested that uronic acid is also involved in development of microbial biofilms at the sediment surface in muddy intertidal systems.

DURA/TDCHO ratio indicates the comparative accounts of production of these compounds. DURA/TDCHO ratio varied from 0 to 0.62 during the period of cultivation. From this result, it is evident that during the stationary growth phase *A. rostrata* produced more amounts of acidic carbohydrates such as uronic acids. The contribution of DURA-C to DOC varied from 0 to 18% during the cultivation period. In natural waters uronic acid yield varied from 2 to 6.3 % (Hung et al., 2005; Khodse et al., 2007). Therefore, DURA production in aquatic environment is useful for ecological and biogeochemical processes.

3A.4.3 Monosaccharide composition of TDCHO

During the growth of *A. rostrata*, cultivation few samples of TDCHO were analysed for monosaccharide composition. The monosaccharide composition of TDCHO provides information about the production of individual monosaccharide during the growth of *A. rostrata*. Fucose was the most abundant monosaccharide contributing about 55 to 75 mol% to the total monosaccharides. Galactose, glucose, mannose, arabinose and xylose were present as minor constituents (Fig. 3A.4). Apparently, this monosaccharide

composition appears to be different from that of the EPS produced by marine planktonic diatoms (Hoagland et al., 1993). For example, rhamnose, fucose and galactose were more abundant monosaccharides found in *Chaetoceros* species (Haug and Myklestad, 1976). Bhosle et al. (1995) found that glucose was most abundant in EPS produced by *Navicula subinflata*. High abundance of rhamnose and small quantities of other sugars were detected in exopolymer produced by *Asterionella socialis* Lewin et Norris (Allan et al., 1972). Decrease in glucose and a relative increase in heteropolysaccharides was found in the TDCHO during the stationary growth phase of *Cylindrotheca closterium* and *Thalassiosira pseudonana* species (Urbani et al. (2005). Monosaccharide differences were observed during the growth phases of *A. rostrata* probably due to nutrient concentrations. For example, dissolved monosaccharide produced by *Cylindrotheca closterium* showed decrease in glucose mol% with increase in other monosaccharides during the cultivation period (Urbani et al., 2005). Fucose concentration increased while other monosaccharides decreased over the period of cultivation. It appears that *A. rostrata* produced fucose rich polymers with the age of culture (Percival, 1970). Decho (1990) reported that production and monosaccharide composition of the EPS produced by microorganism may be influenced by several factors, including growth phase, nutrient concentration, temperature and light condition. Wustman et al. (1998) suggested that fouling diatoms produce extracellular polymers that are highly substituted with sulphate, galactosyl, glucuronosyl and fucosyl residues. A decrease in glucose may be due to the utilization of glucose by respiring cells. Higher fucose and low

glucose polymers linked with uronic acids such as fucoidan polysaccharides are mostly found in brown algae (Nishide et al., 1990).

3A.5 Conclusions

A. rostrata produced more polysaccharides after attaining highest cell density at d 6 of the cultivation period. Similar trend was recorded for DURA. Carbohydrates produced by *A. rostrata* contain 62% of DURA suggesting that polysaccharides were acidic in nature. The fucose was abundant in polysaccharide produced by the diatom. This suggests that *A. rostrata* produced fucose rich acidic polysaccharides. To, the best of our knowledge, this is the first report on the production of DURA by a marine diatom.

Chapter 3B

Differences in carbohydrate profiles in batch culture grown planktonic and biofilm cells of *Amphora rostrata Wm.Sm.*

3B.1 Introduction

Solid surfaces immersed in aquatic environments adsorb dissolved organic matter to condition its surface for colonization by various microorganisms such as bacteria, diatoms, and fungi. Attached microorganisms enmeshed in exopolymeric substances are known as biofilms. Diatoms are amongst the earliest autotrophic colonizers of surfaces immersed in sunlit environments (D'Souza and Bhosle, 2003; Mitbavkar and Anil, 2008; Molino et al., 2009). Diatoms are also known to release extracellular organic compounds in the growth medium and in biofilms that may serve as carbon sources to heterotrophic bacteria (Bahulikar and Kroth, 2008; Bellinger et al., 2009). Therefore the quality and quantity of extracellular organic compounds released by diatoms may influence the microfouling community structure (Decho, 1990; Hoagland et al., 1993; Molino and Wetherbee, 2008).

While attaching to surfaces, diatoms produce extracellular polymeric substances which are rich in carbohydrates. Carbohydrates are common structural and storage products of both biofilm and planktonic diatoms (Cooksey and Wiggesworth-Cooksey, 1995; Smith and Underwood, 1998, 2000; Callow and Callow, 2002; Bellinger et al., 2009). The quality, quantity, and composition of these polymers produced by diatoms are influenced by nutrient status and growth phase (Myklestad, 1977; D'Souza and Bhosle, 2001). Several functions including maintenance, protection and detoxification have been ascribed to diatom polymers. Carbohydrate polysaccharides bind to other organic molecules by different linkages which can be hydrolyzed using treatment with heat or alkali or both (Handa, 1969; Haug and

Myklestad, 1976; Hitchcock, 1977). Therefore, polysaccharides can be isolated using a selective sequential extraction technique (Handa, 1969; Haug and Myklestad, 1976; Hitchcock, 1977; Bhosle et al., 1993).

When grown under normal experimental conditions, some diatom cells attach to culture flask and form biofilms while others remain suspended in culture broth.The diatom cells living in attached mode (biofilm) may have different carbohydrate concentration and composition than those of free-floating (planktonic) forms. Understanding the biochemistry of the polymers produced by diatom under each growth condition is important in enhancing the knowledge of what leads to biofilm development onto surfaces or why organisms maintain a planktonic lifestyle. However, not much is known about the differences, if any, of cellular carbohydrates in attached and planktonic cultures of diatoms. This is because earlier studies on diatoms have mostly focused their attention on characterization of exo or extracellular polysaccharides of planktonic and biofilm cultures of diatoms (Khandeparker and Bhosle, 2001; Faraloni et al., 2003). In contrast, numerous studies have been carried out to evaluate the differences in biochemical metabolites in planktonic and biofilm cells of bacteria (Davey and O'Toole, 2000; Sauer and Camper, 2001; Whiteley et al., 2001; Sauer et al., 2002; Gjersing et al., 2007 and references therein). These studies suggest that there are differences in metabolism of biochemical components including carbohydrates and proteins in biofilm and planktonic cells of bacteria (Davey and O'Toole, 2000; Sauer and Camper, 2001; Sauer et al., 2002; Whiteley et al., 2001; Gjersing et al., 2007 and references therein). Therefore, in the present research, our aims

were to evaluate the total concentrations of cell carbohydrate (TPCHO), TPCHO fractions, neutral carbohydrate (TNPCHO), uronic acid (TPURA), amino sugar (TPAS), and particulate proteins (PP) along with the TNPCHO and TPAS composition of planktonic and biofilm cultures of *A. rostrata* over the period of cultivation, and 2) to identify differences, if any, in the abundances and relative distribution of these compounds in planktonic relative to biofilm cultures.

3B.2 Materials and methods

3B.2.1 Culture conditions and source of algal material

Amphora rostrata Wm. Sm., a pennate diatom was used for this study. The culture was maintained at the Marine Corrosion and Materials Research Division, National Institute of Oceanography, Goa, India. *A. rostrata* was isolated, purified and cultivated following the methods described in chapter 3A.

3B.2.2 Monitoring of growth and chemical and biochemical constituents of the diatom cells

The experiments to assess diatom growth and the production of various chemical and biochemical constituents were carried out in 1 L Erlenmeyer flasks under a light photo-cycle of 12L:12D condition as above. Experimental flasks containing 200 ml of f/2 medium were inoculated with 20 ml of the 5 day old culture (1.8 X 10^5 cells ml^{-1}). The flasks were incubated at room temperature as above to grow planktonic and biofilm cells. The cells which

attached to the culture flask were defined as biofilms, and those remained suspended as planktonic. In order to sample planktonic and biofilm cells, the flasks were removed at 2 d intervals over the period of 10 d. Their contents were decanted to collect the planktonic cells and spent growth medium. The flask containing attached cells was rinsed twice with 15 ml of filtered (0.22 µm mixed cellulose ester filter paper, Millipore) seawater to remove unattached cells. Rinsed seawater containing unattached cells was then transferred to the flask containing planktonic cells and made to a known volume.

The known aliquots (5 to 10 ml) of planktonic cells were individually filtered on pre-combusted (400 °C for 4 h) GF/F glass fiber filter (0.7 µm, Whatman) for the analysis of dry weight (DW), chlorophyll *a* (Chl *a*), particulate organic carbon (POC), PP, TPCHO, dilute acid soluble (Acid-TPCHO), dilute alkali soluble (Alkali-TPCHO) and residual (Res-TPCHO) carbohydrate fractions, TPURA, TNPCHO and TPAS concentration and composition. A small aliquot (1 ml) of planktonic cells were fixed with Lugol's iodine and stored at 4 °C for determination of cell number.

3B.2.3 Removal of attached cells

Diatom cells attached to the culture flask were removed using a soft nylon brush and filtered (0.22 µm) seawater (Bhosle et al., 1989; Khandeparker and Bhosle, 2001; Jain and Bhosle, 2009). The scrapped material was made to a known volume (100 ml) using filtered (0.22 µm) seawater. The known aliquots (5 to 10 ml) of biofilm cells were individually filtered on pre-combusted (400

°C for 4 h) GF/F glass fiber filter (0.7 µm, Whatman) for the analysis of DW, Chl *a*, POC, PP, TPCHO, Acid-TPCHO, Alkali-TPCHO, Res-TPCHO, TPURA, TNPCHO and TPAS. A small aliquot (1 ml) was preserved as above to determine cell count of biofilms. Nutrients (nitrate, phosphate and silicate) data were taken from earlier chapter 3A as and when required.

3B.2.4 Analytical methods

DW of the diatom cells retained on the GF/F filter was determined after drying the filter in an oven at 50 °C for 24 h. The filter was cooled and weighed on a microbalance. The Chl *a*, cell numbers and nutrients were determined following the methods described in chapter 3A. POC was determined by NCS analyzer (CE Instruments, Model-2500), following the method described in chapter 2. PP was extracted from the cells using 0.1 N NaOH, and was analyzed by the bicinchoninic acid method (Smith et al., 1985). TPURAs were analyzed following the method of Filisetti-Cozzi and Carpita (1991) as described in chapter 2.

TPCHOs were estimated by the phenol-sulphuric acid method (Dubois et al., 1956) as described in Khodse et al. (2007). Diatom cells containing GF/F filter was treated with 72% H_2SO_4 at room temperature (28 ±2 °C). After 2 h, 2 ml 5% phenol followed by 5 ml concentrated H_2SO_4 were added. The samples were cooled and the absorption was measured at 490 nm. Glucose was used as standard. Acid-TPCHO, Alkali-TPCHO, and Res-TPCHO carbohydrates were extracted as described earlier (Hitchcock, 1977). The

filter paper containing planktonic or biofilm cells of *A. rostrata* were extracted twice in 4 ml of 0.1 N H_2SO_4 for 2 h. Samples were centrifuged after each acid extraction step, and supernatants were pooled. This was followed by two times extraction with 4 ml of 0.1 N NaOH as described above. Acid and alkali supernatants were concentrated individually and made to a known volume and were analyzed for Acid-TPCHO and Alkali-TPCHO. Res-TPCHO concentration was calculated by subtracting the sum of the concentrations of Acid-TPCHO and Alkali-TPCHO carbohydrates from the TPCHO concentration. TNPCHO concentration and composition of the planktonic and biofilm *A. rostrata* cells were determined using the capillary gas chromatographic method following the details given in chapter 2.

TPASs were determined following the method of Zhang and Amelung (1996). The samples were hydrolyzed with 6 M HCl for 5 h at 105 °C. The samples were cooled, neutralized with 0.4 M KOH, and TPASs were converted to aldononitrile derivatives (Guerrant and Moss, 1984), and analysed by the GC-Mass spectrometer (MS). A GC-MS system (Shimadzu Model-GC-MS QP-2010) with electron impact ionization mode (70 ev) and a capillary column (30 m, 0.32 mm i.d. 0.25 µm df) was used. The injector temperature was 250 °C, and the oven temperature was programmed from 120 °C to 250 °C at 10 °C/min and held for 2.5 min and again increased to 270 °C at 20 °C/min and maintained for 2 min. The interface and ion source temperatures of the MS were maintained at 280 °C and 250 °C, respectively.

Sum of all the indentified amino sugars has been defined as total AS. Analytical reproducibility of total amino sugar by GC-MS method was ± 7%.

3B.2.5 Statistical analysis

Analysis of variance (ANOVA) was carried out to understand the variations of POC, Chl *a*, TPCHO, TNPCHO, TPAS, TPURA and PP in biofilm and planktonic cultures of *A. rostrata*. Data of these components of the biofilm and planktonic cultures collected over the 10 d period of cultivation were used to perform ANOVA analysis. The ANOVA analysis was performed using Microsoft excel software loaded on personal computer.

3B.3 Results

3B.3.1 Changes in DW, Chl a, and diatom cell counts

After few hours following inoculation, *A. rostrata* cells started attaching to the culture flask. The Chl *a* concentration of both the planktonic and biofilm *A. rostrata* cells increased over the cultivation period, and reached a maximal value on d 6 and d 8, respectively, following inoculation (Fig. 3B.1a). Thereafter, a small decrease in Chl *a* concentration was observed for both cell types. A similar trend was observed for the cell count of the planktonic and biofilm cells of the diatom (Fig.3B.1b). Growth rate was relatively higher for biofilm cells than plaktonic cells (Fig. 3B.1c). For the planktonic cells, DW increased with the cultivation period, and reached a maximal value on d 6 (Fig. 3B. 1d), and then decreased for the remaining period of cultivation. In contrast, DW of the biofilm cells increased over the period of cultivation (Fig.

3B.1d). Concentrations of nutrients in the culture medium declined rapidly over the period of cultivation.

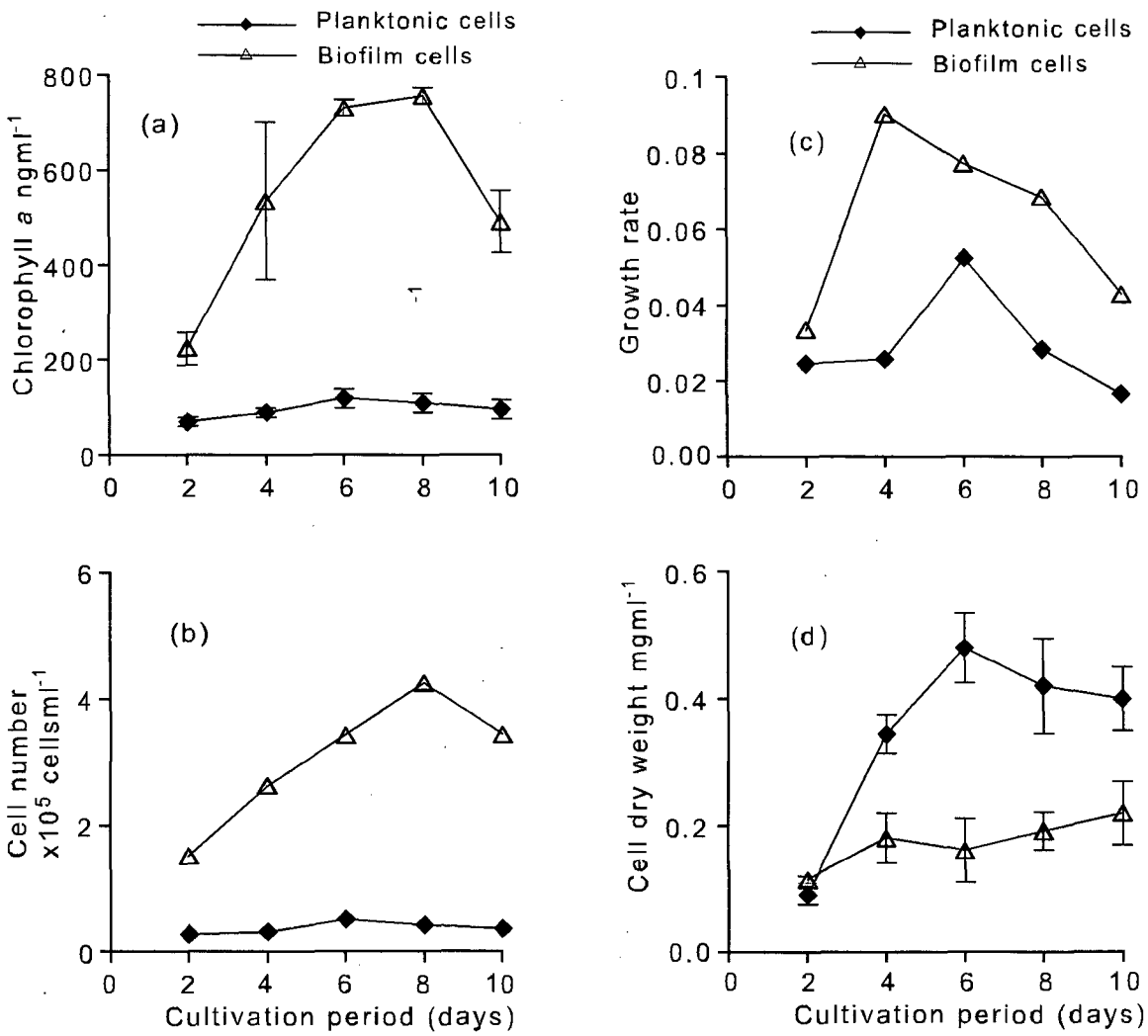


Fig.3B.1. Changes in Chlorophyll *a* (a), cell numbers (b), growth rate (c), and cell dry weight (d) for planktonic and biofilm cells of *A. rostrata* over the period of cultivation.

3B.3.2 POC, particulate carbohydrates and protein profile

In order to compare POC, particulate carbohydrates and PP profile of the planktonic and biofilm cells, concentrations of POC, PP, TPCHO, TNPCHO, TPURA and TPAS were normalized to cell numbers (Fig. 3B.2). Concentrations of POC, PP and TPCHO generally increased over the period of cultivation in both planktonic and biofilm cultures of *A. rostrata* (Fig. 3B.2). Concentrations of POC, PP, and TPCHO were significantly different (Table 3B.1), and were 2 to 5 times greater in planktonic as compared to the biofilm cultures (Fig. 3B.2a, b, c). For the planktonic diatom cells, concentrations of PP showed some decrease after 6 d period of cultivation (Fig. 3B.2b).

Table 3B.1. One way analysis of variance (ANOVA) to assess changes in chlorophyll *a* (Chl *a*), particulate organic carbon (POC), total particulate carbohydrate (TPCHO), particulate protein (PP), total neutral particulate carbohydrate (TNPCHO), particulate amino sugars (TPAS) and uronic acids (TPURA) in planktonic and biofilm cells of *A. rostrata.*

Parameter	Chl *a*	POC	TPCHO	PP	TNPCHO	TPAS	TPURA
MS	3.38	474.31	153.86	28.00	38.64	0.02	7.09
F	27.57	48.30	13.89	27.63	4.36	6.56	4.38
p-value	**0.001**	**0.001**	**0.006**	**0.001**	0.070	**0.034**	0.069

Values shown in bold indicate significant differences.

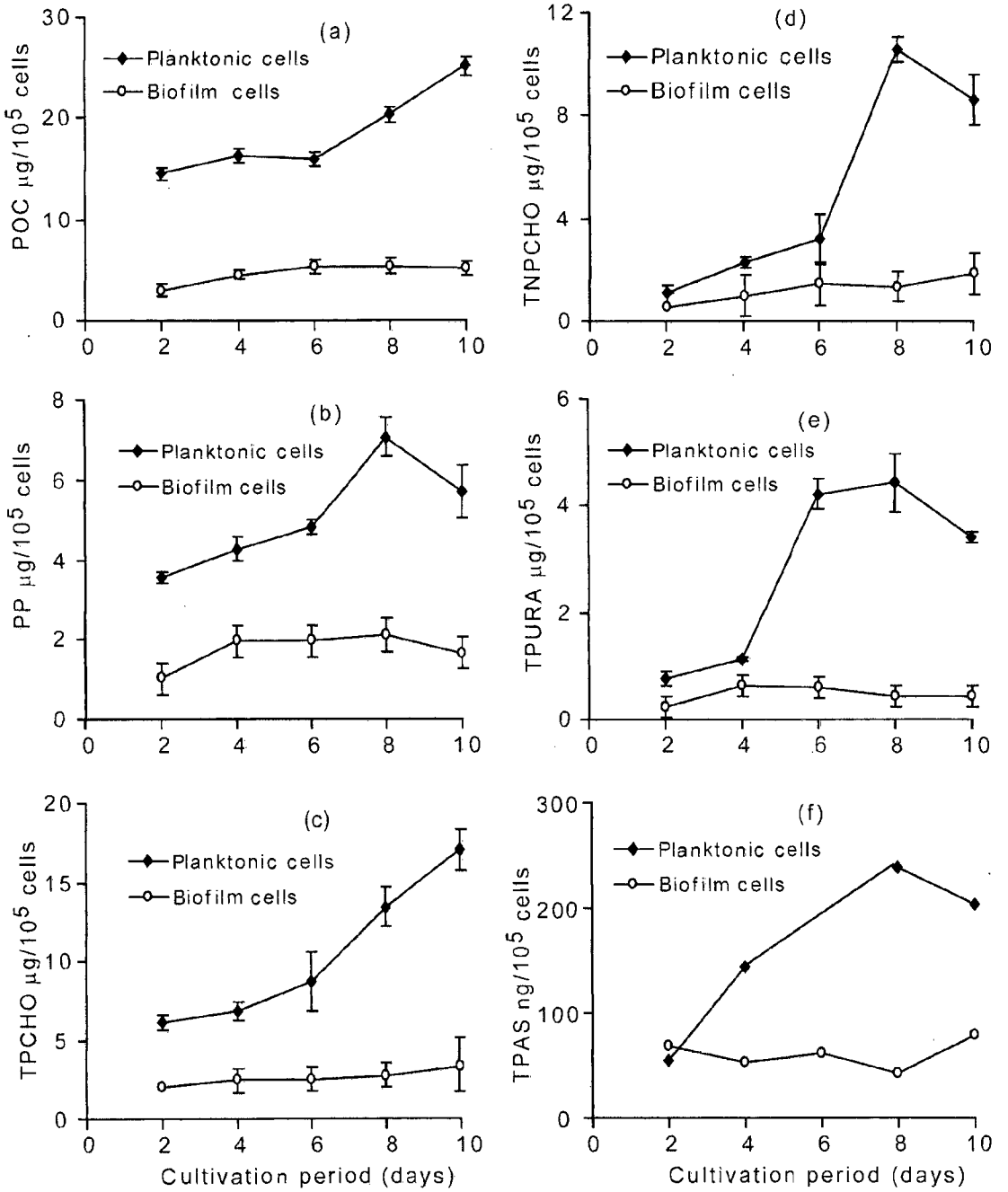


Fig. 3B.2. Concentration of particulate organic carbon (POC) (a), particulate protein (PP) (b), total particulate carbohydrate (TPCHO) (c), total neutral particulate carbohydrate (TNPCHO) (d), total particulate uronic acids (TPURA) (e), and total particulate amino sugars (TPAS) (f) in planktonic (—◆—) and biofilm (—o—) cells of *A. rostrata*.

Table 3B.2. Changes in TPCHO/Chl *a*, PP/Chl *a*, PP/TPCHO, and TPURA/TPCHO ratios in planktonic and biofilm cells of *A. rostrata*.

Cultivation period (days)	TPCHO/Chl *a* ratio	PP/Chl *a* ratio	PP/TPCHO ratio	TPURA/TPCHO ratio
Planktonic cells				
2	24.46	14.29	0.58	0.13
4	24.32	15.18	0.62	0.16
6	37.73	20.90	0.55	0.48
8	51.40	27.01	0.53	0.33
10	65.75	21.95	0.33	0.20
Biofilm cells				
2	13.27	6.94	0.52	0.11
4	11.87	9.61	0.81	0.26
6	11.79	9.21	0.78	0.24
8	15.44	11.85	0.77	0.15
10	23.37	11.60	0.50	0.13

TPCHO = total particulate carbohydrates; PP = particulate protein; Chl *a* = chlorophyll *a*; TPURA = total particulate uronic acid.

3B.3.3 TPCHO/Chl a, PP/Chl a and PP/TPCHO ratios

For both the planktonic and biofilm cells of *A. rostrata,* TPCHO/Chl *a,* and PP/Chl *a* ratios generally increased over the period of cultivation, with the ratios being higher for planktonic cells (Table 3B.2). PP/TPCHO ratio was higher in biofilm cells, and generally decreased in both planktonic and biofilm cultures over the period of cultivation (Table 3B.2). A higher TPURA/TPCHO ratio was noted in planktonic cells, which generally decreased in both cell types.

3B.3.4 Carbohydrate fractions

Concentrations of Acid-TPCHO, Alkali-TPCHO and Res-TPCHO generally increased over the period of cultivation in both planktonic and biofilm cells, and were higher in the former cells (Table 3B.3).

Table 3B.3. Dilute acid soluble (Acid-TPCHO), dilute alkali soluble (Alkali-TPCHO) and residual (Res-TPCHO) particulate carbohydrates and their contribution to total particulate carbohydrates (TPCHO) in planktonic and biofilm cells of *A. rostrata*.

Cultivation period (days)	Acid-TPCHO		Alkali-TPCHO		Res-TPCHO	
	a	b	a	b	a	b
Planktonic cells						
2	3.93 ±0.00	64.20	1.11 ±0.00	18.00	1.08	18.00
4	4.80 ±0.55	70.12	1.09 ±0.13	12.91	0.96	16.94
6	6.49 ±0.93	74.54	1.24 ±0.10	14.28	0.97	11.18
8	9.12 ±0.64	67.78	1.09 ±0.21	8.13	3.24	24.09
10	10.90 ±1.00	63.89	1.14 ±0.20	6.68	5.02	29.44
Biofilm cells						
2	0.85 ±0.00	43.23	0.51 ±0.00	25.90	0.60	30.92
4	1.16 ±0.09	47.89	0.51 ±0.10	20.91	0.75	31.22
6	1.17 ±0.16	51.80	0.55 ±0.02	24.29	0.79	23.91
8	1.68 ±0.31	61.38	0.50 ±0.03	18.06	0.56	20.55
10	2.33 ±0.40	69.86	0.63 ±0.03	18.81	0.38	11.32

a = concentration expressed as $\mu g/10^5$ cells;

b = percentage of TPCHO.

When expressed as a percentage of TPCHO, Acid-TPCHO did not vary much in planktonic cells. In contrast, for the biofilm cells, the contribution of Acid-TPCHO to the TPCHO increased from 43% at d 2 to 70% at d 10 following inoculation (Table 3B.3). For both cell types, Alkali-TPCHO contribution to TPCHO decreased over the period of cultivation. Res-TPCHO contribution to TPCHO increased in planktonic cells and decreased in the biofilm cells (Table 3B.3).

3B.3.5 TNPCHO concentration and composition

Capillary gas chromatographic analysis detected the presence of arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose and xylose in the planktonic and biofilm cells (Table 3B.4). For the planktonic cells, concentrations of TNPCHO increased over the 8 d, and then decreased (Fig. 3B.2d). In contrast, TNPCHO concentrations in biofilm cells showed consistent increase over the period of cultivation, however TNPCHO concentrations were not significantly different in these two cell types (Table 3B.1).

For planktonic cells, the abundance (as mol %) of glucose, rhamnose, ribose, xylose decreased while that of fucose, mannose and galactose increased through time (Table 3B.4). In contrast, abundance of glucose increased, whereas that of fucose, ribose, arabinose, and xylose decreased in the biofilm cells over the period of cultivation. Overall, glucose and fucose distribution in planktonic and biofilm cells showed contrasting trends (Table 3B.4).

Table 3B.4. Monosaccharide composition (Mol %) of planktonic and biofilm cells of *A. rostrata.*

tivation	Monosaccharides (Mol %)								Group composition (Mol %)		
iod ys)	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Hexo	Pento	Deoxy-Hexo
nktonic cells											
2	7.32[a]	9.61[a]	1.05[a]	3.47[a]	6.52[a]	8.14[a]	10.15[a]	53.75[a]	72.03	11.04	16.93
4	3.46 ±0.01	9.79 ±0.02	3.13 ±0.00	2.65 ±0.00	5.67 ±0.01	5.86 ±0.01	27.42 ±0.04	42.12 ±0.07	75.40	11.45	13.25
6	1.80 ±0.30	31.52 ±3.06	0.58 ±0.09	0.75 ±0.08	5.13 ±1.01	12.87 ±0.01	22.20 ±0.37	25.03 ±4.18	60.10	6.46	33.32
8	1.46 ±0.11	37.10 ±5.49	0.73 ±0.03	5.09 ±0.45	3.62 ±0.97	13.50 ±0.45	21.49 ±2.82	17.00 ±3.60	52.00	9.44	38.56
10	1.90 ±0.13	38.44 ±5.00	0.56 ±0.09	7.77 ±1.17	2.63 ±0.52	13.89 ±2.10	24.36 ±1.97	10.45 ±1.20	48.70	10.95	40.34
ilm cells											
2	4.34[a]	16.00[a]	3.06[a]	2.22[a]	6.60[a]	9.95[a]	22.51[a]	35.88[a]	68.34	11.89	20.33
4	3.75 ±0.99	11.42 ±1.30	4.95 ±0.91	0.66 ±0.14	4.11 ±0.66	6.81 ±1.52	31.72 ±4.04	36.57 ±5.56	75.10	9.72	15.17
6	2.47 ±0.07	6.27 ±0.16	1.82 ±0.56	3.01 ±0.90	2.63 ±0.21	3.60 ±0.09	22.57 ±1.13	57.53 ±4.38	83.69	7.47	8.75
8	2.46 ±0.24	7.56 ±1.22	1.59 ±0.13	0.48 ±0.09	4.01 ±0.70	3.36 ±0.01	19.40 ±1.15	61.14 ±2.12	83.91	6.08	10.01
10	3.84 ±0.34	6.56 ±0.19	1.26 ±0.16	0.38 ±0.05	3.26 ±0.50	3.36 ±0.17	21.25 ±1.89	60.75 ±3.81	85.36	4.91	10.41

Hexo = Hexoses (mannose + galactose + glucose); Pento = Pentoses (ribose + arabinose + xylose); Deoxy-Hexo = Deoxy hexoses (rhamnose + fucose). a = Single observation as cells were very less.

When monosaccharides were classified into hexoses (glucose, galactose and mannose), pentoses (ribose, xylose and arabinose) and deoxyhexoses (rhamnose and fucose), hexoses decreased while pentoses and deoxyhexoses increased in planktonic cells with the cultivation period (Table 3B.4). In the biofilm cells, concentrations of hexoses increased while that of pentoses and deoxyhexoses decreased over the cultivation period (Table 3B.4).

3B.3.6 TPURA concentration

As observed for the most of the other components, concentrations of TPURA were higher in the planktonic cells and lower in the biofilm cells of *A. rostrata* (Fig. 3B.2e). For the planktonic cells, the TPURA concentration was highest at d 6, and then decreased. In contrast, biofilm TPURA concentration showed small but consistent increase over the period of cultivation. However, the observed differences in the TPURA concentration in the planktonic and biofilm cells were not significant ($p > 0.05$) (Table 3B.1). The TPURA/TPCHO ratio varied from 0.13 to 0.48 and from 0.11 to 0.26 in planktonic and biofilm cells, respectively (Table 3B.2).

3B.3.7 TPAS concentration and composition

Glucosamine (GlcN), galactosamine (GalN) and mannosamine (ManN) were detected in both planktonic and biofilm cells of *A. rostrata*. Total TPAS concentrations increased over the period of cultivation for the planktonic cells (Fig. 3B.2f). Whereas for the biofilm cells, TPAS concentration was greater at

3B.4 Discussion

3B.4.1 Cell growth and concentrations of carbohydrates and proteins

The observed increase in DW, Chl *a,* and cell numbers indicated growth of both planktonic and biofilm cells of *A. rostrata* (Fig. 3B.1). Concentrations of Chl *a*, POC, PP, and TPCHO, in planktonic and biofilm cells showed significant differences ($p < 0.001$). Diatoms are known to produce greater amounts of carbohydrates in culture (Handa,, 1969; Myklestad, 1989), and in natural waters, particularly during the stationary growth phase and/or under nutrient deficient conditions (Myklestad, 1977; Ittekkot et al., 1981; Tanoue and Handa, 1987). This was also the case with planktonic and biofilm cells of *A. rostrata.* However, the production of TPCHO and PP by the planktonic cells was higher than the biofilm cells (Fig. 3B.2). This was also evident from the higher TPCHO/Chl *a* and PP/Chl *a* ratios for the planktonic cells. Production of TPCHO by diatoms is influenced by nutrient concentrations in the growth medium (Myklestad, 1977; D'Souza and Bhosle, 2001; Underwood et al., 2004). Concentrations of nitrate, phosphate and silicate in the culture medium decreased over the period of cultivation. Nutrient deficient condition may be responsible for the higher TPCHO/Chl *a* rato in planktonic cells. In contrast, biofilm cells of *A. rostrata* had low TPCHO/Chl *a* ratio suggesting relatively low production of TPCHO by the biofilm cells probably due to nutrient sufficient condition (Sekar et al., 2002). The PP/TPCHO ratio generally decreased with incubation period for both cell types (Table 3B.2). However, PP/TPCHO ratio was generally higher (0.50 to 0.81) for the biofilm

cells as compared to the planktonic cells (0.33 to 0.62) (Table 3B.2), implying that biofilm cells produced greater amounts of PP relative to TPCHO. During the growth of diatoms, protein synthesis appears to dominate during the initial exponential growth phase, while amounts of cellular protein decrease with increasing nutrient reduction (Myklestad, 1977; Mayzaud et al., 1990; Underwood et al., 2004; Diekmann et al., 2009). As compared to the planktonic cells PP/TPCHO ratio was higher in biofilms. Higher PP/TPCHO ratio suggests that nutrients were not depleted, and protein production was still dominant in biofilms of *A. rostrata.*

3B.4.2 Carbohydrate fractions

In diatoms, carbohydrate polymers bind to organic molecules by different linkages. Hydrogen and van der Waals bonds that bind organic macromolecules with each other and with cell-surfaces can be dissociated by heating (Handa, 1969; Haug and Myklestad, 1976; Hitchcock, 1977). Ester linkages are involved in binding of polysaccharides to other organic molecules while carbohydrates and proteins are bound to each other by *O*-glycosidic bonds. Both ester and *O*-glycosidic bonds are easily destroyed by alkaline treatment (Collins and Ferrier, 1995). Therefore, selective sequential extraction technique often has been applied to extract carbohydrates with specific linkages from diatoms (Handa and Tominaga, 1969; Handa and Yanagi, 1969; Hitchcock, 1977). When biofilm and planktonic cells were extracted with dilute acid and alkali, there were apparent differences in the relative distribution of TPCHO fractions in these two types of cells (Table

3B.3). Relatively greater abundance of Alkali-TPCHO indicates higher concentration of TPCHO with *O*-glycosidic and ester bonds in the biofilm cells of the diatom (Table 3B.3). This suggests that polysaccharides with ester and *O-glycosidic* bonds probably were involved in the development of biofilms of *A. rostrata* on glass surface. Carbohydrate polymers play important role in attachment of diatoms to surfaces (Bahulikar and Kroth, 2008). It appears that alkali extracted polysaccharides were responsible for the cell to cell attachment (Wustman et al., 1997; Wigglesworth-Cooksey et al., 2001; Bahulikar and Kroth, 2008). Furthermore, it has been observed that firmly attached diatoms on glass slides can be removed only after addition of hot bicarbonate solution (Bellinger et al., 2005).

It was also interesting to note that the concentrations of dilute acid extractable TPCHO for the first 6 d following inoculation were lower for the biofilm cells as compared to the planktonic cells (Table 3B.3). This suggests that polysaacharides that bind to other organic molecules by hydrogen and/or van der Waals bonds were not produced in large quantities by the biofilms of *A. rostrata.* This probably was expected because *A. rostrata* cells need to produce carbohydrates that bind to other organic molecules with stronger ester and O-glycosidic bonds so as to form biofims.

3B.4.3 TNPCHO concentration and composition

Production of TNCHO by both the planktonic cells of *A. rostrata* increased over the period of cultivation (Fig. 3B.2d). Glucose was the most abundant monosaccharide in both the planktonic and biofilm cells (Table 3B.4). Glucose

can account for 20 to 80 mol % of TNPCHO during diatom blooms in natural environments and in pure culture grown in laboratory (Handa and Yanagi, 1969; Mykalestad, 1974; Ittekkot et al., 1982; Tanoue and Handa, 1987; Bhosle et al., 1990; D'Souza and Bhosle, 2001). Many diatom species are also known to produce glucose rich storage polysaccharide such as β-1,3 glucan (Handa and Tominaga, 1969; Bhosle et al., 1995; Granum and Myklestad, 1999; Storseth et al., 2004; Diekmann et al., 2009). However, it was interesting to note that the relative distribution of glucose and fucose in planktonic and biofilm cells was very different. Abundance of glucose (as mol %) decreased rapidly in planktonic cells over the period of cultivation probably suggesting its utilization by the respiring cells of *A. rostrata.* Decrease in glucose mol % has been recorded for the respiring cells of diatoms (Handa and Yanagi, 1969; Hitchcock, 1977; Tanoue and Handa, 1987). The consistent depletion of glucose was accompanied by an increase in fucose during the stationary phase of growth of the planktonic cells of *A. rostrata* (Table 3B.4).This suggests that fucose substitutes for glucose during stationary phase of growth of the planktonic cells of *A. rostrata.* It appears that fucose content increased with age or growth or estuarine conditions in some diatoms (Hecky et al., 1973; Bahulikar and Kroth, 2008). Moreover, in some diatoms, glucose rich polysaccharides are replaced by fucose rich polysaccharides during the stationary phase of growth of some diatoms (Percival, 1970). Roszkowski et al. (1989) have reported that fucose rich polysaccharide inhibits the adhesion process of metastatic sarcoma L-1 cells in lungs of mice. In view of this, it is possible that fucose rich carbohydrates

are produced by the planktonic cells of *A. rostrata* perhaps to prevent their adhesion to glass surface.

Concentrations of TNPCHO, especially glucose, increased in *A. rostrata* biofilms over the period of cultivation, indicating the production of glucose rich polysaccharides by the biofilms of *A. rostrata*. The pads, capsules and stalk are enriched in glucose rich adhesive polysaccharides. These polysaccharides are involved in keeping cells together and attachment of cells to surface substratum (Underwood et al., 1995; Decho, 2000; Molino and Wetherbee, 2008; Bahulikar and Kroth, 2008). Moreover, attached cells spend less energy for locomotion and metabolic requirements. As a result, glucose exhibits different trends in planktonic and biofilm cells of *A. rostrata*.

When grouped together as hexoses, pentoses and deoxy hexoses, abundance of hexoses decreased in planktonic and increased in biofilm cell of *A. rostrata* (Table 3B.4). Temporal variation in hexoses in both cell types was mostly controlled by the changes in glucose concentration. As discussed above glucose abundance decreased in planktonic cells and increased in biofilm cells of *A. rostrata* indicating different metabolic pattern for this monosaccharide. Similarly, contrasting trend was also recorded for the distribution of deoxy sugars (rhamnose plus fucose) in biofilms and planktonic cells of the diatom. This again demonstrates the metabolic differences in the distribution of these sugars in planktonic and biofilm cells of the diatom.

3B.4.4 TPURA concentration

TPURAs are carboxylated sugars which may influence the adhesion of microorganisms to glass coupons (Daniel et al., 1987; Decho, 1990; Hoagland et al., 1993; Garg et al., 2009; Jain and Bhosle, 2009). Moreover, these acidic carbohydrates are also involved in immobilization, diatom cell aggregation, sedimentation, cell adhesion to surfaces, detoxification of toxic compounds, adsorption of dissolved organic matter, and removal of metal ions (Decho, 1990; Mopper et al., 1995; Tang et al., 2001; Jain and Bhosle, 2009). Distribution of TPURA in planktonic and biofilm cells of *A. rostrata* over the period of cultivation yet again points to the fact that these two cell types followed different metabolic trends.

TPURA/TPCHO ratio was higher in planktonic cells as compared to the biofilm cells suggesting that TPURA accounted for a greater proportion of carbohydrates in planktonic cells (Table 3B.2). Higher TPURA/TPCHO ratio in biofilms at d 4 following inoculation suggests that TPURAs were produced during the initial stages of adhesion of *A. rostrata* cells to glass surface. High TPURA/TPCHO ratio in planktonic cells at d 6 and 8 coincide with decrease in cell numbers probably indicating their involvement in cell aggregation and sedimentation.

3B.4.5 TPAS concentration and composition

GlcN and GalN are important constituents of diatom cells (Benner and Kaiser, 2003) and adhesive structures (pads, stalk and capsules) formed by diatoms during attachment (Bahulikar and Kroth, 2008). GlcN concentrations were 2 to

3 times greater in biofilm cells than the planktonic cells of *A. rostrata* (Table 3B.5). This implies that more GlcN was produced by the biofilm cells of the diatom (Table 3B.5). Storage polymers are preferentially degraded as compared to the structural polymers (Ittekkot et al., 1982; Hernes et al., 1996; Unger et al., 2005). Preferential removal of GlcN may be responsible for the relatively lower concentrations in planktonic cells. It is hypothesized that nutrients, especially nitrate influence the relative distribution of GlcN and GalN. Under nitrate depleted condition, abundance of GlcN decreased while that of GalN increased in two marine bacteria when they were grown under nitrate depleted condition (Khodse and Bhosle, unpublished results). It is possible that some of these factors may be responsible for the observed differences in TPAS concentrations and composition in planktonic and biofilm cells of *A. rostrata*.

3B.5 Conclusions

A. rostrata cells when grown under batch culture, showed clear differences in concentrations of TPCHO, PP, Acid-TPCHO and Alkali-TPCHO, Res-TPCHO, TNPCHO, and TPAS concentration and composition. However, further studies using different diatom cultures are needed to better understand the production of biochemical compounds in planktonic and biofilm cells. Such studies may be of value in developing techniques to control diatom biofilms on industrially useful structures.

Chapter 4A

Dynamics of dissolved carbohydrates and uronic acids in the Mandovi estuary, west coast of India

4A.1 Introduction

Dissolved organic carbon (DOC) is one of the largest reactive components of the organic carbon in marine waters (Romankevich, 1984; Benner et al., 1992). As a result, DOC plays an important role in global carbon cycle. The composition of DOC is not completely characterized. However, carbohydrates account for a large fraction (~10 to 85 %) of DOC in seawater (Thurman, 1985; Pakulski and Benner, 1994) and pore water (Burdige et al., 2000). This is because carbohydrates are ubiquitous and present in aquatic environments as structural cell components (e.g., cellulose, pectin and chitin), storage polymers (e.g., starch), algal and bacterial exudates (e.g., uronic acids and sulfate esters) and other biopolymers such as lipopolysaccharides (Romankevich, 1984; Decho and Lopez, 1993). Primary production is a dominant source of carbohydrates in deep ocean environments. These compounds are also released from various other sources including direct algal release due to auto-lysis, extracellular excretion due to sloppy feeding and egestion by zooplankton, microbial degradation of organic matter, viral or bacterial lysis of phytoplankton cells. Moreover, in coastal and estuarine environments, dissolved carbohydrates are also derived from excretion by macrophytes, periphyton, allochthonous DOM from rivers and soil. Dissolved carbohydrates serve as carbon source for heterotrophic bacteria. Heterotrophic uptake and secondary production influence the abundance of dissolved carbohydrates in aquatic environments.

Dissolved carbohydrates are analyzed without hydrolysis and after hydrolysis using dilute-HCl (0.09 N) (Burney and Sieburth, 1977). Using

this method structural polysaccharides are not effectively hydrolyzed (Pakulski and Benner, 1992, 1994). Such hydrolysis treatment, therefore, may underestimate the concentrations of dissolved carbohydrates in seawater samples. In order to overcome this problem, Pakulski and Benner (1992) recommended the use of concentrated H_2SO_4 for the complete hydrolysis of structural polysaccharides. Application of this method produced carbohydrates yields that are equal or greater than those obtained using the method of Burney and Sieburth (1977) and Parsons et al. (1984a). Thus the improved method of hydrolysis with concentrated H_2SO_4 produced more accurate determination of total dissolved carbohydrates. Moreover, the application of the method to seawater samples helps us to understand the dynamics of hitherto unknown class of dissolved carbohydrates called as acid resistant polysaccharides. Therefore, the application of the modified method of Pakulski and Benner (1992) to estuarine samples will provide a better picture of carbohydrates distribution in estuarine waters.

In addition to neutral carbohydrates, bacteria, fungi, algae, plants and other organisms produce acidic carbohydrates such as uronic acids. These compounds can account for ~ 4 to 30 %, and for 0.7 to 5 % of TDCHO and DOC in river and estuarine waters, respectively (Hung et al., 2001, 2005). Concentration of uronic acids generally decreased with increasing water column depth suggesting their utilization by heterotrophic organisms (Khodse et al., 2007). It appears that uronic acids are less degradable as compared to carbohydrates (Hung et al., 2003). Uronic acids play an important role in many marine processes such as humic acid

formation, detoxification of toxic chemicals and removal of metal ions, production of macroaggregates, microbial adhesion and biofilm development (Yamaoka, 1983; Decho, 1990; Mopper et al., 1995; Santschi et al., 1998; Jain and Bhosle, 2009). Despite their importance in the above processes, little is known about their distribution, sources and cycling in marine and estuarine waters (Hung et al., 2001, 2003, 2005; Khodse et al., 2007, 2008).

Most of the above studies on TDCHO species including DURA have been carried out in temperate, Arctic and Antarctic waters. In contrast, numerous studies have been carried out to understand physical, chemical and biological processes in the tropical Mandovi estuary (Qasim and Sen Gupta 1981; De Sousa 1983; Devassy and Goes 1988; Ansari and Parulekar 1993; Shetye et al., 1995, 2007; Ram et al., 2003; Partihary et al., 2009). However, little is known about biochemical characterization of dissolved organic matter in tropical waters, particularly in estuarine waters. Therefore, our aims were, 1) to understand the distribution of various dissolved carbohydrate species and uronic acids in the Mandovi estuary, and 2) to evaluate the role of some factors in controlling the distribution of TDCHO and DURA in the Mandovi estuary.

4A. 2 Materials and methods

4A.2.1 Study area

The Mandovi estuary is located in Goa, on the west coast of India between 15° 15' N and 15° 42' N and 73° 45' E and 74° 24' E (Fig. 4A.1). The Mandovi River originates in the Western Ghats of India and flows into the

Arabian Sea. The total length of the estuary is about 75 km and the width at the mouth is about 3.2 km. The climate is humid, tropical with moderate temperature. The Mandovi river transports 6 $\times 10^{15}$ cm^3/year of water to the Arabian Sea (Suprit and Shankar, 2008). Ninety percent of this transport occurs during the monsoon season (June – September).

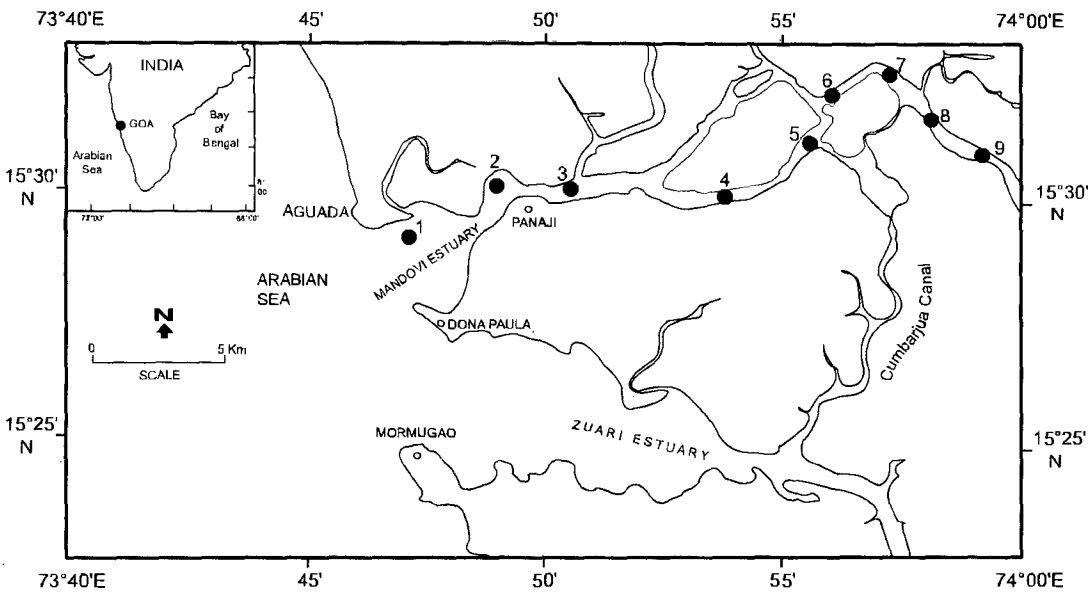


Fig. 4A.1. Sampling locations in the Mandovi estuary, west coast of India.

The estuary is fed largely by the monsoon precipitation and receives a large amount of land runoff from the ore mining areas and forest land. In contrast, the non-monsoon period, the input of fresh water is negligible and is regulated by the semidiurnal tides. The residence time of the water in the Mandovi estuary is 5–6 d during the monsoon season and about 50 d during in the nonmonsoon seasons (October–May) (Qasim and Sen Gupta, 1981). Physical, chemical and biological aspects of the Mandovi estuary have been recently reviewed (Shetye et al., 2007).

4A.2.2 Sample collection

Surface (~1 m) water samples were collected from 9 locations in the Mandovi estuary during monsoon (August 2005) and pre-monsoon (March 2007) season (Fig. 4A.1). Water samples were collected using 5 liter Niskin samplers. Immediately after collection, water was passed through 200 µm nylon screen to remove zooplankton. A sub-sample was filtered through mixed cellulose ester filter papers (0.22 µm, Millipore). This filtrate was defined as dissolved fraction. Filtered samples were preserved at -20 °C until analysis. For Chl *a* analysis water sample was filtered through a Whatman GF/F filter. The filter was immediately transferred to a clean screw capped glass vial and transported to the laboratory in an ice box. For the phytoplankton cell counts, one liter water sample was preserved with a few drops of Lugol's iodine (1% w/v). In order to estimate total bacterial count (TBC), seawater sample (20 ml) was fixed with glutaraldehyde (final concentration 2%) and stored at 4 °C until analysis.

4A.2.3 Analytical methods

Sea surface temperature and surface salinity were measured by the portable conductivity / temperature /depth (CTD) (Model SBE 19 plus, Sea-Bird Electronics, USA). Dissolved oxygen (DO) was fixed and analyzed following the Winkler's method (Parsons et al., 1984). Dissolved nitrate, phosphate and silicate were estimated spectrophotometrically (Parsons et al., 1984). Chl *a* was extracted overnight in 90% acetone and estimated following the method of Parsons et al. (1984). To estimate phytoplankton cells count and composition, one liter sample was brought

down to 20 to 25 ml using settling and siphoning procedure (Prabhu Matondkar et al., 2007). One milliliter of the concentrated sample was transferred in Sedgewick-Rafter plankton counting chamber. Cells were counted under inverted Olympus microscope (200X magnification).

4A.2.4 Total bacterial count (TBC)

TBC was estimated following the acridine orange staining method (Parsons et al., 1984). A known volume of seawater (2 to 5 ml) was stained with acridine orange (final concentration 0.01%) for 5 min, filtered onto 0.22 µm black Nuclepore polycarbonate filter. The filter was then placed on a drop of fluorescence free oil, another drop of oil placed onto it and covered with a cover slip. Bacterial cells were counted at least 25 randomly selected fields using 100X objective, and an epifluorescence microscope (Nikon). Average cell number per field was calculated, and used to estimate total bacterial cells following the procedure described by Parsons et al. (1984).

4A.2.4.1 Bacterial production (BP)

Bacterial production was estimated from the incorporation rate of ^{3}H-thymidine. The samples were analyzed following the JGOFS protocol (1994). Ten milliliter unfiltered water sample was added into 20 ml scintillation vial. To this, 30 µl (15 nM) of the working solution of thymidine was added. Similarly, one set of sample was fixed with filtered formalin (4% final concentration) before the addition of ^{3}H-thymidine and treated as control. Both sample and the control vials were incubated in dark for 60

min at room temperature. At the end of the incubation period ^{3}H-thymidine uptake was terminated with the addition of filtered formalin (4 % final concentration). The sample was filtered at low pressure through 0.22 µm cellulose nitrate (Millipore company) filter. The filter paper was then rinsed two times with 5% ice-cold trichloroacetic acid (TCA), followed by three rinses of ice-cold ethanol. After complete dryness, filter was transferred to a clean scintillation vial and completely dissolved in 2 ml ethyl acetate. Four ml of scintillation cocktail were added to each sample and allowed to stabilize overnight. The samples were counted using a scintillation counter (Model Wallac-1209).

The thymidine uptake rate was calculated using the following equation:

(methyl-^{3}H-thymidine) nmole $l^{-1}h^{-1}$ = $(DPM_{sample} - DPM_{blank})/2200$ x (1000/V) x (1/SA) x (60/T)

DMP = disintegrations per minute, V = volume ml, SA = specific activity,

T = incubation time (min).

The thymidine uptake rate was converted to bacterial cells using the conversion factor (CF) of 2 x 10^{18} cells per mole of thymidine utilized (Riemann et al. 1987) . Bacterial cells were converted to bacterial carbon production (BP) using a conversion factor of 15 fgc per bacterial cell (Coron et al., 1995).

4A.2.5 Dissolved organic carbon (DOC)

DOC was analyzed by the high temperature (680 °C) oxidation method using a Shimadzu TOC-5000 carbon analyzer. Sample values were

corrected for the instrument blank (Benner and Strom, 1993). Potassium hydrogen phthalate was used as a standard (50 µM C to 400 µM C). Eight samples were analyzed between injection of Milli-Q water and working standard solution. Analytical variation of this method was ± 3.1%.

4A.2.6 Dissolved carbohydrates and uronic acids analysis

MCHOs were determined without hydrolysis by the 2,4,6 tripyridyl -s-triazine (TPTZ) spectrophotometric method of Myklestad et al. (1997). To estimate HCl-hydrolyzable dissolved carbohydrates (HCl-TDCHO) water sample was hydrolyzed with HCl (0.09N) at 100 °C for 20 h and the librated MCHOs were determined by the TPTZ method as described in chapter 2. In order to estimate total dissolved carbohydrates (TDCHO), water sample (10 ml) was concentrated to dryness under rotary vacuum evaporator, and then treated with 1 ml of 12 M H_2SO_4 for 2 h at room temperature (28 ±2 °C) (Bhosle et al., 1998). Subsequently, the sample was diluted to 1.2 M using Milli-Q water and hydrolyzed at 100 °C for 3 h in an oven. Librated free MCHOs were determined by the TPTZ method as described in chapter 2. Dissolved polysaccharide (DPCHO) concentrations were calculated by subtracting MCHO from TDCHO. HCl-hydrolyzable dissolved polysaccharides (HCl-DPCHO) were estimated by subtracting MCHO concentrations from HCl-TDCHO concentrations (Pakulski and Benner, 1992, 1994). Hydrolysis-resistant polysaccharides (HR-DPCHO) were estimated by subtracting the concentrations of HCl-TDCHO from the concentrations of TDCHO (Pakulski and Benner, 1992, 1994; Bhosle et

al., 1998). The concentrations of DURA were analyzed by the method of Filisetti-Cozzi and Carpita (1991) as described in chapter 2.

4A.2.7 Monosaccharide composition of TDCHO

Concentration and composition of total dissolved neutral sugars were determined by HPLC (Pakulski and Benner, 1992; Broch and Kirchman, 1997). Ten milliliter of 0.22 µm filtered estuarine water was dried using a rotary vacuum evaporator. The dried sample was treated with 12 M H_2SO_4 for 2 hours at room temperature, diluted with Milli-Q water to 1.2 M H_2SO_4, flushed with nitrogen gas, sealed and hydrolyzed for 3 h. After cooling, internal standard (2-deoxyribose) was added, the sample was then neutralized and partially desalted using pre-combusted $CaCO_3$. Sample was centrifuged at 3000 rpm for 10 min. and the supernatant was collected. The supernatant was desalted using a mixed bed of anion (Dowex 1-X8) and cation (Dowex-50W-X8) exchange resins. The desalted sample was reduced to a known volume.

Neutral sugars were analyzed by the HPLC system consisting of a metal free quaternary gradient pump (Dionex GS50) and ED40 electrochemical detector (Model no. 5200A ESA), and a manual injector with 20 µl sample loop. The detector was equipped with an electrochemical cell containing a gold working electrode and Ag/AgCl reference electrode. Monosaccharides were detected by measuring the electrical charge generated by their oxidation at the surface of a gold electrode. Settings of the detector pulse potentials and durations were. E1 = 50 mv, t1 = 400 ms; E2 = 500 mv, t2 = 300 ms; E3 = -100 mv, t3 = 400 ms. The response time was set to 1 second and output range was 50 nA.

The sugars were separated using 4 x 250 mm PA-1 column (Dionex CarboPack -1) packed with nonporous polymeric resin with an anion exchange capacity of 100 μeq and the CarboPak PA guard column. NaOH (1.5 mM) was used as a mobile phase at a flow rate of 1.0 ml/min. The mobile phase was prepared using freshly prepared UV-Milli-Q water and the semiconductor grade sodium hydroxide containing low amount of carbonate (< 1% Na_2CO_3, Sigma Chemical Company). The mobile phase was degassed with nitrogen for 20 min. After each sample run, the column PA-1 was washed with 200 mM NaOH for 10 min, and then the re-equilibration of the column was carried out with 1.5 mM NaOH for 10 min to obtain consistent retention times of the desired compounds. Identification and quantification of the sample compounds were done using standard sugars (Sigma) and the data handling System Gold Software (Beckman).

4A.2.8 Statistical analysis

One-way ANOVA was carried out to understand spatial and seasonal variations in Chl *a*, DOC, TDCHO, MCHO, DPCHO and DURA using STATISTICA program (5) loaded in the personal computer. Correlation between parameters was assessed using Excel software program (Sokal and Rohlf, 1981).

4A.3 Results

4A.3.1 General hydrography

The surface water temperature was low (27.4 to 28.3 °C) in the monsoon, and high (30.7 to 31.8 °C) during the pre-monsoon season (Fig. 4A.2).

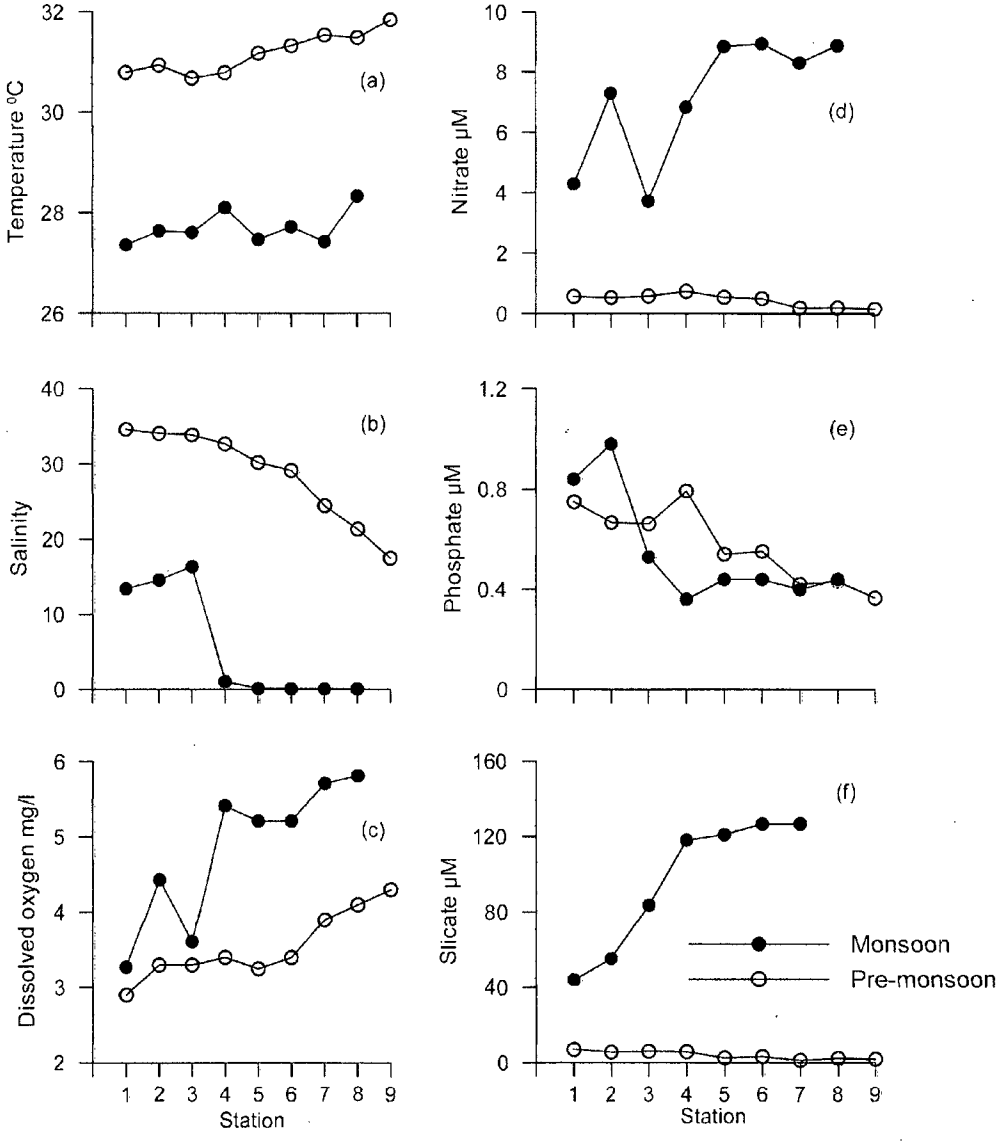


Fig. 4A.2. Variation of temperature (a), salinity (b), dissolved oxygen (c), nitrate (d), phosphate (e) and silicate (f) in surface waters of the Mandovi estuary during the monsoon and pre-monsoon seasons.

Although no particular trend was noticed, water temperature was higher at the upstream station # 8 and lower at station #1 near the mouth of the estuary (Fig. 4A.2a). Strong seasonal and spatial variations were also recorded in salinity of the surface waters (Fig. 4A.2b). During the wet season of monsoon the estuary is partially stratified in the bottom layer and nearly salt free in the upstream regions. The DO concentration varied from 2.9 to 5.8 mg l^{-1} in surface waters of the Mandovi estuary (Fig. 4A.2c), and the concentration was higher during the monsoon period. The phosphate, nitrate and silicate concentration in surface waters varied from 0.37 to 0.98 µM, 1.18 to 8.94 µM and 1.2 to 126.9 µM, respectively (Fig. 4A.2d,e,f). During monsoon concentrations of nitrate and silicate were higher than those observed in pre-monsoon (Fig. 4A.2d,f).

4A.3.2 Phytoplankton biomass and composition

The concentrations of Chl *a* in surface waters showed significant seasonal variations, and the values varied from 1.0 to 17.24 µg l^{-1} during the study period (Fig. 4A.3a). The phytoplankton cells density in surface waters varied from 0.26 × 10^4 (# 6) to 3.84 × 10^4 (Station # 7), and from 0.27 × 10^4 to 3.21 × 10^4 in the monsoon and pre-monsoon season, respectively (Fig. 4A.3b). High cell count recorded at station #7 during the monsoon season was due to dinoflagellates bloom. Both Chl *a* and the phytoplankton cell numbers were higher during the pre-monsoon than that observed for the monsoon season (Fig. 4A.3).

contributed 58 to 98% and dinoflagilates 2 to 42 % to the total phytoplankton community (Fig. 4A.4).

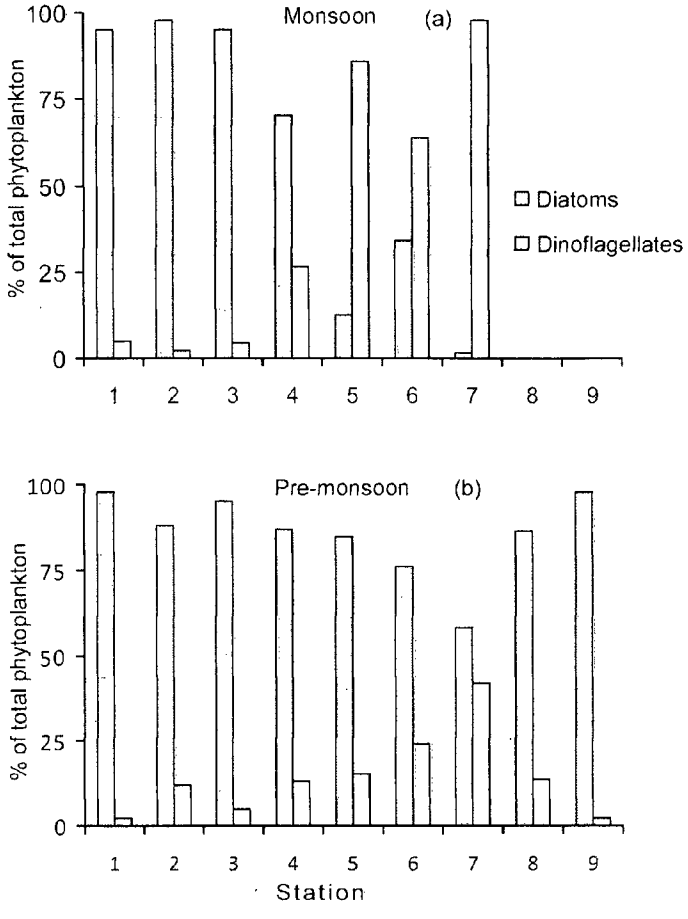


Fig. 4A.4. Relative distribution of diatoms and dinoflgellates in surface waters of the Mandovi estuary during the monsoon and pre-monsoon seasons.

4A.3.3 Total bacterial count (TBC) and bacterial production (BP)

TBC in surface waters varied from 5.4 × 10^8 to 42.1 × 10^8 cells l^{-1} (Fig. 4A.5a). TBC was low in monsoon and high in the pre-monsoon season. During the pre-monsoon period, total bacterial production ranged from 2.7 nM C $l^{-1}h^{-1}$ (station #1) to 48.0 nM C $l^{-1}h^{-1}$ (station #7) (Fig. 4A.5b).

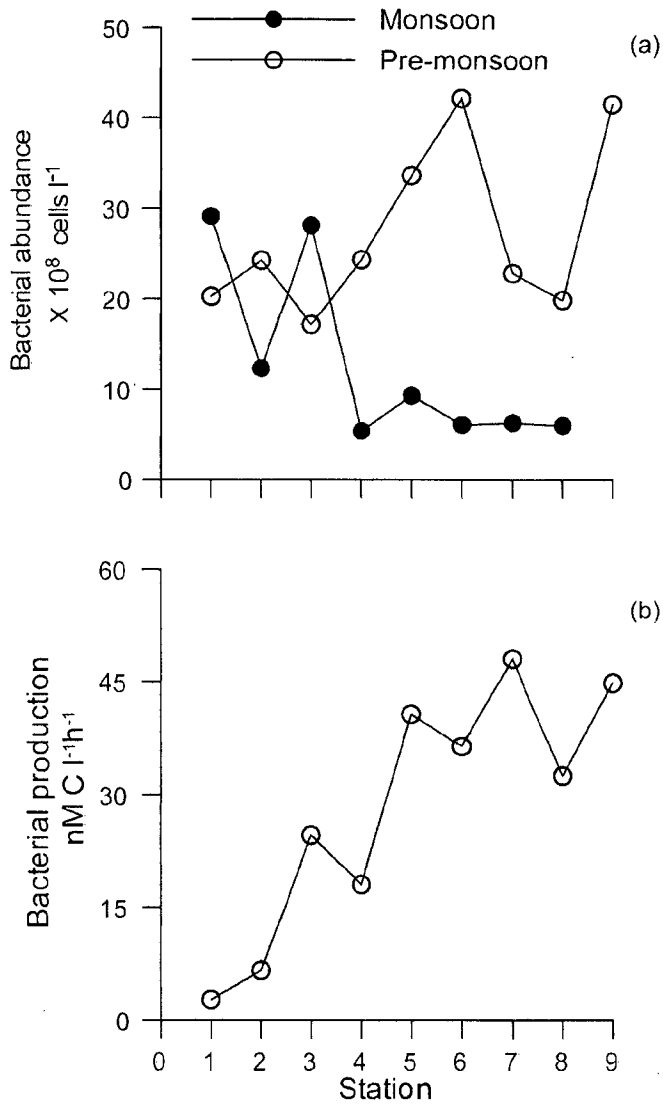


Fig. 4A.5. Seasonal variability of bacterial abundance (a) and bacterial production (b) in the surface water of the Mandovi estuary.

4A.3.4 Distribution of DOC

In the surface waters of the Mandovi estuary DOC concentration showed spatial and seasonal variations, and ranged between 80 µM C and 195 µM C during the study period (Tables 4A.1, 4A.2). During the monsoon season, DOC concentrations of surface waters decreased from the mouth to the upstream region of the estuary (Table 4A.1). During monsoon

season average DOC concentration was lower (138 ±41) as compared to the DOC concentration (174 ±18) during pre-monsoon. During both the seasons DOC concentration did not follow the trends observed for Chl *a* in the surface waters.

4A.3.5 Distribution of TDCHO, HCl-TDCHO

The distribution of TDCHO and its constituent fractions at various locations of the Mandovi estuary is shown in Table 4A.1. Concentrations of TDCHO and HCl-TDCHO in the surface waters of the Mandovi estuary ranged between 17.7 and 67.3 µM C, and from 10.3 to 29.0 µM C, respectively (Table 4A.1). For both the seasons, highest concentration of TDCHO was observed at station # 5. TDCHO concentrations showed significant (p<0.008) seasonal variation (Table 4A.2).

4A.3.6 Distribution MCHO

MCHO concentration showed significant seasonal variations (4.1 to 15.4 µM C) (Table 4A.1, 4A.2). Average MCHO concentration was higher in the pre-monsoon (9.5 µM C) than the monsoon season (5.6 µM C). Station #8 during the monsoon and stations #5 and #8 during the pre-monsoon had higher concentration of MCHO. In the surface waters MCHO contributed 8 to 24% (average 12.6 ±5.2%), 21.3 to 48.3% (average 32.7 ±9.42) to the TDCHO during the monsoon and the pre-monsoon seasons, respectively (Table 4A.1). MCHO/DPCHO ratio varied from 0.08 to 0.93 in the Mandovi estuary (Fig. 4A.6). During the monsoon, the ratio was low (0.08 to 0.3;

average 0.14) while it was high (0.27 to 0.93; average 0.51) in the pre-monsoon season (Fig. 4A.6).

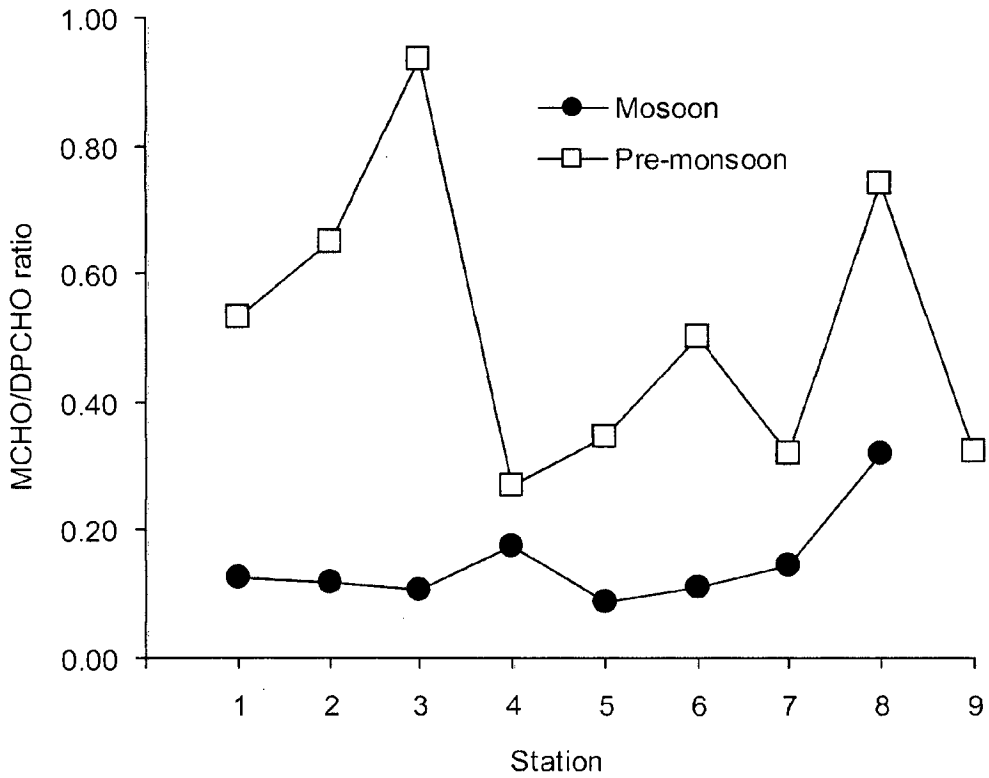


Fig.4A.6. Variation of MCHO/DPCHO ratio during monsoon and pre-monsoon in the Mandovi estuary (MCHO: monosaccharide, DPCHO : dissolved polysaccharides).

A.1. Distribution of dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), HCl-hydrolyzable total dissolved carbohydrate (HCl-TDCHO), free charide (MCHO), dissolved polysaccharides (DPCHO), HCl-hydrolyzable dissolved polysaccharides, (HCl- DPCHO), HCl-resistant dissolved polysaccharides (HR- and dissolved uronic acids (DURA) in the Mandovi estuary.

DOC	TDCHO	HCl-TDCHO		MCHO			DPCHO		HCl-DPCHO			HR-DPCHO			DURA	
a	a	a	b	a	b	c	a	b	a	b	c	a	b	c	a	b
195	56.8±5.2	21.2±0.4	37.4	6.3±0.2	11.0	12.4	50.5±5.5	89.0	15.0±1.9	26.4	29.7	35.5±1.7	62.6	70.3	6.9±0.3	12.1
181	51.3±5.7	12.1±1.2	23.7	5.4±0.9	10.5	11.7	45.9±2.8	89.5	6.8±0.7	13.2	14.7	39.2±4.2	76.3	85.3	5.7±0.2	11.1
167	43.3±2.6	16.4±1.2	37.8	4.1±0.6	9.5	10.5	39.2±3.5	90.5	12.3±0.4	28.3	31.3	26.9±3.8	62.2	68.7	4.5±0.2	10.4
145	37.5±3.6	11.2±1.1	29.9	5.6±0.9	14.9	17.5	31.9±4.7	85.1	5.6±0.4	15.0	17.7	26.3±2.6	70.1	82.3	4.7±0.4	12.5
118	67.3±8.0	18.8±1.1	27.9	5.4±0.8	8.0	8.7	62.0±7.6	92.0	13.4±1.0	19.9	21.7	48.5±5.3	72.1	78.3	6.7±0.4	9.9
80	47.9±4.3	21.5±0.2	45.0	4.8±0.7	10.0	11.1	43.1±5.1	90.0	16.8±2.3	35.0	38.9	26.4±2.7	55.0	61.1	3.4±0.2	7.1
103	41.9±3.6	19.1±0.2	45.7	5.3±0.8	12.6	14.5	36.6±3.0	87.4	13.8±1.7	33.0	37.8	22.8±1.5	54.3	62.2	2.3±0.2	5.5
111	33.5±3.1	15.8±1.3	47.1	8.1±0.5	24.3	32.1	25.4±2.8	75.7	7.6±2.1	22.8	30.1	17.7±1.0	52.9	69.9	5.0±0.3	14.9
138±41	47.4±11.0	17.0±3.9	36.8±8.9	5.6±1.2	12.6±5.2	14.8±7.5	41.8±11.4	87.4±5.2	11.4±4.2	24.2±7.9	27.7±8.9	30.4±10.0	63.2±8.9	72.3±8.9	4.9±1.6	10.5±3.0
soon																
167	23.0±4.0	15.8±1.7	68.6	8.0±1.0	34.8	53.3	15.0±4.0	65.2	7.8±1.7	33.8	51.8	7.2±0.7	31.4	48.2	7.7±0.2	33.6
148	21.5±3.3	11.8±1.0	54.8	8.5±1.1	39.4	65.0	13.0±2.5	60.6	3.3±0.6	15.4	25.5	9.7±0.5	45.2	74.5	3.7±0.6	17.3
156	17.7±0.8	10.3±1.0	58.4	8.5±1.3	48.3	93.5	9.1±2.3	51.7	1.8±0.1	10.1	19.6	7.3±1.0	41.6	80.4	3.2±0.2	18.2
172	35.3±3.5	14.4±1.5	40.7	7.5±1.0	21.3	27.0	27.8±7.4	78.7	6.9±1.0	19.4	24.7	21.0±1.2	59.3	75.3	8.5±0.4	24.1
194	40.8±2.3	15.7±1.1	38.5	10.5±0.8	25.8	34.7	30.3±2.8	74.2	5.2±1.1	12.7	17.2	25.1±1.8	61.5	82.8	9.2±0.4	22.4
192	28.8±3.8	14.6±0.5	50.8	9.6±0.6	33.4	50.1	19.2±4.2	66.6	5.0±0.4	17.4	26.1	14.2±0.8	49.2	73.9	7.0±0.6	24.3
181	35.4±3.0	15.5±0.9	43.6	8.5±1.2	24.1	31.8	26.9±4.8	75.9	6.9±2.1	19.5	25.7	20.0±3.4	56.4	74.3	10.8±0.5	30.6
160	36.4±1.9	29.0±2.8	79.7	15.5±1.0	42.6	74.2	20.9±2.1	57.4	13.5±1.7	37.1	64.6	7.4±0.7	20.3	35.4	8.8±0.4	24.2
193	36.8±2.0	12.5±0.3	34.1	9.0±0.9	24.5	32..5	27.8±3.1	75.5	3.5±1.1	9.5	12.7	24.2±2.0	65.9	87.3	9.4±0.7	25.5
174±18	30.6±8.2	15.5±5.4	52.1±15	9.5±2.4	32.7±9.4	51.3±22.6	21.1±7.5	67.3±9.4	6.0±3.4	19.5±9.8	29.8±17.0	15.1±7.5	47.9±15.0	70.2±17.1	7.6±2.6	24.5±5.2

µM C; b = percentage of TDCHO; c = percentage of DPCHO; NS = no sample

4A.3.7 Dissolved polysaccharides DPCHO, HCl-DPCHO and HR-DPCHO

In the Mandovi estuary, TDPCHO concentrations showed significant ($p<0.0029$) seasonal variations (Table 4A.2), and varied from 9.1 to 62.0 μM C (average 30.8 ±14.1 μM C) (Table 4A.1). These DPCHO concentrations were 6 to 8 times higher than those recorded for the MCHO. DPCHO accounted for 76 to 92 % (average 87.4 ±5.2 %), and 52 to 79 % (average 67.3 ±9.4 %) of the TDCHO during the monsoon and pre-monsoon season, respectively (Table 4A.1). Within the polysaccharide pool, concentrations of HCl-DPCHO varied from 5.6 to 16.8 μM C (average 11.4 ±4.2 μM C) in the monsoon and from 1.8 to 13.5 (average 6.0 ±3.4 μM C) in the pre-monsoon season (Table 4A.1). HCl-DPCHO contributed from 14.7 to 38.9 % and from 12.7 to 64.6 % of DPCHO in the monsoon and pre-monsoon season, respectively (Table 4A.1). Concentrations of HR-DPCHO ranged between 17.7 and 48.5 μM C (average 30.4 ±10.0 μM C) in monsoon, and from 7.2 to 25.1 μM C (average =15.1 ± 7.5 μM C) in pre-monsoon season (Table 4A.1). HR-DPCHO was a major fraction of the DPCHO pool and accounted for 61.1 to 85.3 % (average 72.3 ± 8.9 %), and 35.4 to 87.3 % (average 70.2 ±17.1%) of the DPCHO in the monsoon and pre-monsoon season, respectively. Similarly, the contribution of HR-DPCHO to the TDCHO was higher in the monsoon (52.9 to 76.3 %; average 63.2 ±8.9 %) than in the pre-monsoon (20.3 to 65.9 %; average 47.9 ±15.0 %) season (Table 4A.1).

4A.3.8 Dissolved carbohydrate yields

TDCHO accounted for ~26 to 59 %, and ~11 to 23 % of DOC during the monsoon and pre-monsoon season, respectively (Fig. 4A.7a). The contribution of MCHO to DOC varied from 2.47 to 7.32 % and 4.38 to 9.73 % during the monsoon and pre-monsoon season, respectively (Fig. 4A.7c). The contribution of TDCHO to DOC was relatively high (~40 to 59 %) during the monsoon season, especially at stations #5, #6, and #7 (Fig. 4A.7). Yields of most of the TDCHO fractions were higher during the monsoon season and lower in pre-monsoon season (Fig. 4A.7).

In the Mandovi estuary, bacterial cell numbers showed a significant negative correlation with TDCHO:DOC ratio (Fig. 4A.9a). The surface water TDCHO:DOC ratio had significant negative correlation with the situ water temperature (Fig. 4A.9b).

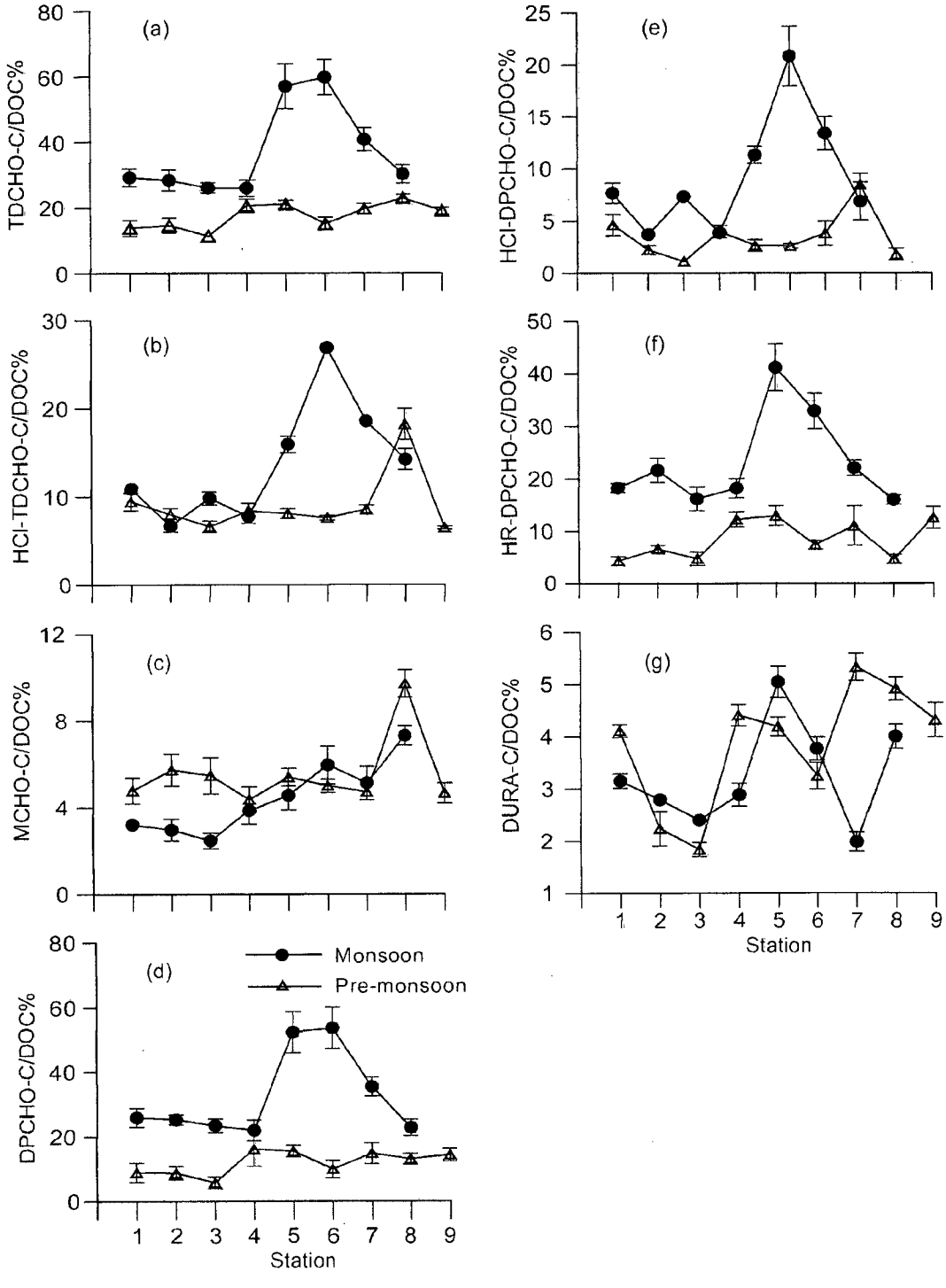


Fig. 4A.7. Variation in the yields as (%) of TDCHO-C/DOC (a), HCl-TDCHO-C/DOC (b), MCHO-C/DOC (c), DPCHO-C/DOC (d), HCl-DPCHO-C/DOC (e), HR-DPCHO-C/DOC (f) and DURA-C/DOC (g) in monsoon and pre-monsoon in the Mandovi estuary. Bar represent the ± SD.

4A.3.9 Monosaccharide composition of TDCHO

HPLC analysis revealed the presence of fucose, rhamnose, arabinose, galatose, glucose, xylose, mannose and ribose in the surface waters of the Mandovi estuary. Of these glucose was most abundant fraction (37.7 to 50.6 %), followed by fucose (7.5 to 24 %), galactose (8.9 to 16.6), rhamnose (3.7 to 15.2 %), arabinose (2.7 to 18%), xylose (ND to 14 %), mannose (ND to 9.7%) and ribose (ND to 8%) in surface DOM (Fig. 4A.8).

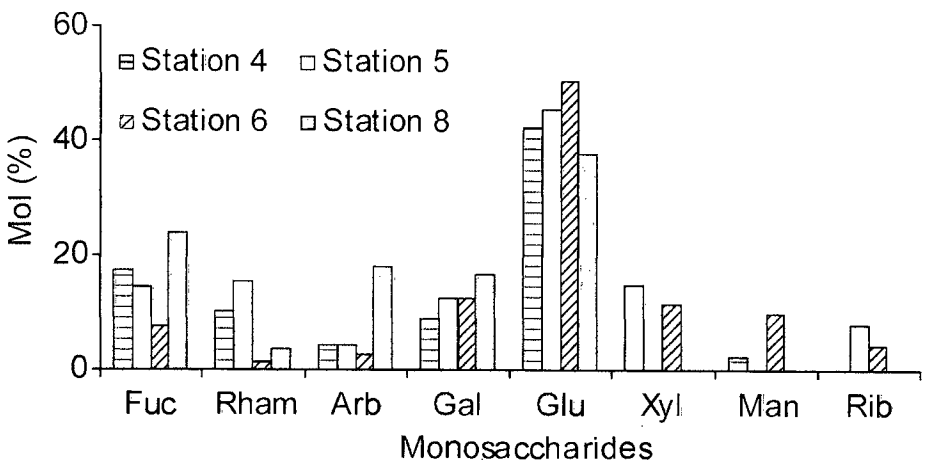


Fig. 4A.8. Monosaccharide composition (as mol %) of the dissolved total carbohydrates (TDCHO) at some stations in the Mandovi estuary during the monsoon season (Fuc, fucose; Rham, rhamanose; Arb, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Rib, ribose).

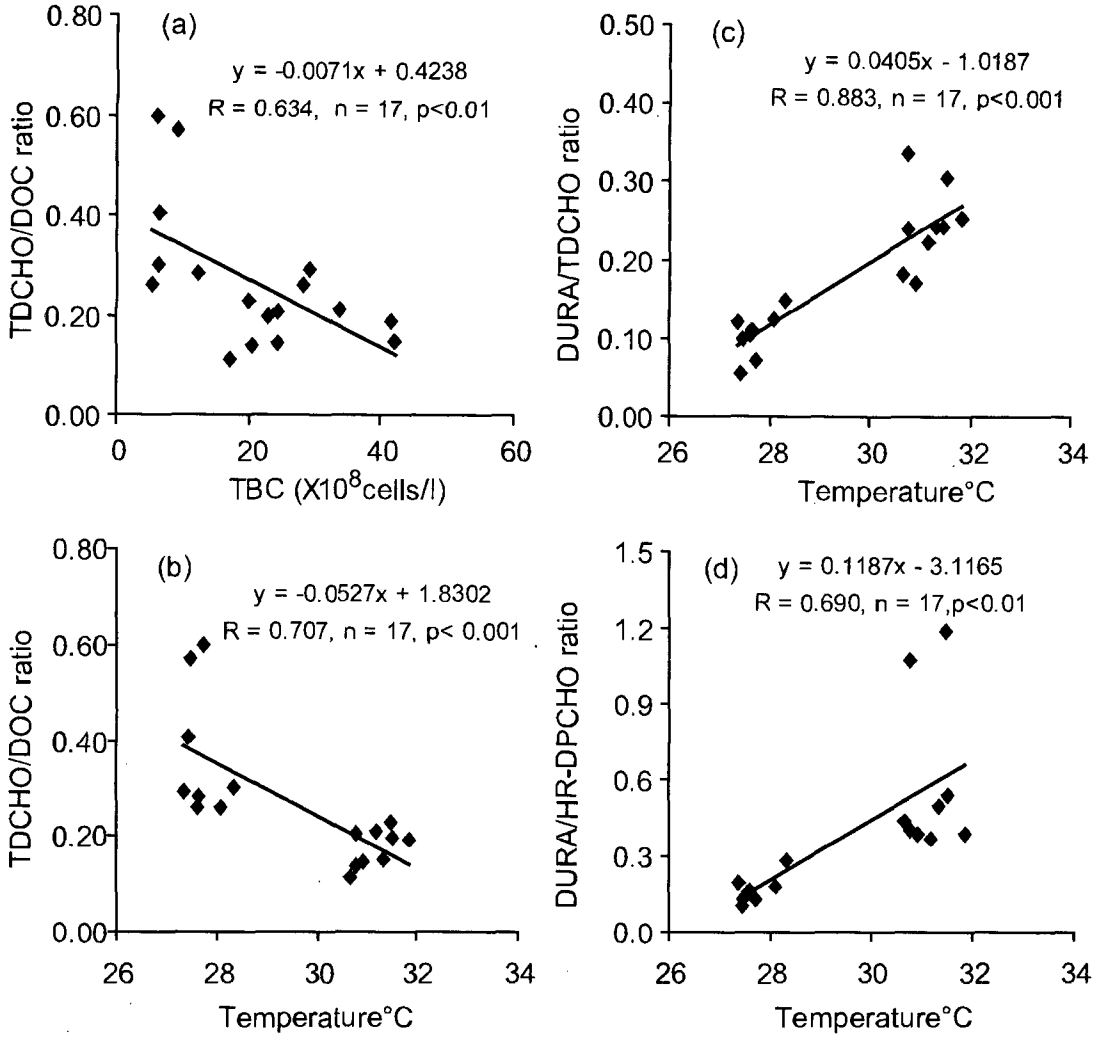


Fig. 4A.9 Relationship of TBC with TDCHO/DOC ratio (a), and the temperature with TDCHO/DOC ratio (b), DURA/TDCHO ratio (c),and DURA/HR-DPCHO ratio (d) in surface water of the Mandovi estuary.

4A.3.10. Dissolved uronic acids (DURA)

In the Mandovi estuary, DURA showed seasonal variations (Table 4A.1), and varied from 2.3 to 10.8 µM C (Table 4A.1). Average DURA concentrations were higher in pre-monsoon (7.6 ±2.6) than those recorded for the monsoon (4.9 ±1.6). Although the concentrations of DURA varied seasonally, the contribution DURA-C to DOC did not vary much during both the monsoon (~2 to 5 %; average 3.25 ±0.98 %) and the pre-

monsoon (~2 to 5 %, average 3.84 ±1.17 %) seasons (Fig. 4A.7g). The *situ* water temperature showed significant positive correlation with DURA:TDCHO and DURA:HR-DPCHO ratio (Fig. 4A.9 c,d)

Table 4A.2 One-way ANOVA of the spatial (between stations) and seasonal variation in Chl *a*, DOC, TDCHO, MCHO, DPCHO and DURA in the Mandovi estuary.

Source of variation	df	Seq SS	Adj MS	F	*p*-value	F crit
Chl *a*						
Station	7	0.005	0.001	1.002	0.4988	3.787
Season	1	0.034	0.034	46.050	0.0003	5.591
Error	7	0.005	0.001			
Total	15	0.044				
DOC						
Station	7	3441.729	491.676	0.336	0.9132	3.787
Season	1	4547.535	4547.535	3.109	0.1212	5.591
Error	7	10238.568	1462.653			
Total	15	18227.832				
TDCHO						
Station	7	657.890	93.984	0.979	0.5107	3.787
Season	1	1233.551	1233.551	12.853	0.0089	5.591
Error	7	671.826	95.975			
Total	15	2563.266				
MCHO						
Station	7	43.958	6.280	3.607	0.0561	3.787
Season	1	63.141	63.141	36.264	0.0005	5.591
Error	7	12.188	1.741			
Total	15	119.287				
DPCHO						
Station	7	687.985	98.284	1.107	0.4485	3.787
Season	1	1854.857	1854.857	20.886	0.0026	5.591
Error	7	621.662	88.809			
Total	15	3164.504				
DURA						
Station	7	27.310	3.901	0.697	0.6769	3.787
Season	1	24.539	24.539	4.386	0.0745	5.591
Error	7	39.167	5.595			
Total	15	91.016				

Chl *a*; DOC; TDCHO; MCHO; DPCHO; DURA same as in table 4A.1

4A.4. Discussion

4A.4.1. General hydrography and nutrient

During the monsoon season, nearly fresh water condition exists in the estuary because of the heavy precipitation. In contrast, the estuary experiences nearly marine condition during the dry pre-monsoon season because of negligible fresh water discharge. DO concentrations increased from station #1 at the mouth of the estuary to the station #8 in the upstream region of the estuary. Elevated temperature results in increased respiration rates leading to low oxygen concentrations during the pre-monsoon. Whereas, low temperature in monsoon results in increased gas solubility leading to higher DO concentrations.

Generally, concentrations of nitrate and silicate were higher at the freshwater end and lower at the mouth of the estuary. In contrast, phosphate concentration showed opposite trend. During the monsoon season, salinity showed significant inverse relationships with nitrate ($r = -0.814$, $n = 8$, $p<0.01$), and silicate ($r = -0.746$, $n = 8$, $p<0.02$) suggesting that freshwater discharge was a major source for these nutrients. Mining rejects also form an important source of nitrate in the estuary (de Souza, 1999). Qasim and Sen Gupta (1981) reported that the decrease in nitrate concentration from head to mouth in the Mandovi estuary was due to the utilization of the nitrate for the photosynthetic activity. Salinity showed significant positive relationship with phosphate concentrations during both the monsoon ($r = 0.856$, $n = 8$, $p<0.01$) and the pre-monsoon ($r = 0.910$, $n = 9$, $p<0.001$) seasons. From these relationships, it was evident that seawater was a source of phosphate in the

estuary. Phosphates can also be released into the water from bottom sediment through vertical and horizontal mixing by wind, tides and bottom current. During the pre-monsoon season, DO concentrations showed fairly good negative correlations with the concentrations of phosphate ($r = -0.843$, $n = 9$, $p<0.001$), silicate ($r = -0.779$, $n = 9$, $p<0.01$) and to some extent nitrate ($r = -0.351$, $n = 9$, $p>0.05$) suggesting in situ biological control.

Higher nutrient concentrations were associated with lower phytoplankton cell counts and the Chl *a* concentrations during the monsoon season probably because of the light limited condition due to the presence of high suspended particulate matter (Prabhu Matondkar et al., 2007). Higher TBC associated with higher Chl *a* and DOC concentration during the pre-monsoon season suggesting that phytoplankton biomass influenced bacterial abundance. BP showed significant positive relationship with DOC ($r= 0.711$, $n=9$, $p<0.02$), TDCHO ($r= 0.678$, $n=9$, $p<0.02$), and surface water temperature ($r= 0.788$, $n=9$, $p<0.01$). This implies that the bacterial production and heterotrophic activity in the Mandovi estuary was regulated by temperature and some labile compounds like dissolved carbohydrates.

4A.4.2. Distribution of DOC

DOC concentrations recorded for surface waters are in the range of values reported earlier for the Mandovi estuary, other estuaries and coastal waters (Lobbes et al., 2000; Kohler et al., 2003; Ram et al., 2003; Hung et al., 2003, 2005). DOC concentrations of surface waters decreased from the mouth to the upstream region of the estuary (Table 4A.1).

Though DOC is produced by biological processes, there was no significant correlation between Chl *a* and DOC during both the monsoon and pre-monsoon seasons, indicating that phytoplankton biomass was not the major factor controlling the concentrations of DOC in the Mandovi estuary (Table 4A.1). However, DOC concentrations showed significant positive relationships with the TBC during both the monsoon ($r = 0.756$, $n = 8$, $p<0.01$), and pre-monsoon ($r = 0.811$, $n = 9$, $p<0.01$) seasons. Similarly, during the pre-monsoon season, DOC concentrations also showed significant positive correlation with the bacterial productivity ($r = 0.711$, $n = 9$, $p<0.02$). This implies the importance of DOC in controlling the distribution of bacteria in the Mandovi estuary (Amon and Benner, 2003). Furthermore, phytoplankton production coupled with microbial and grazing activities often control DOC concentrations in aquatic environments (Strom et al., 1997; Hopkinson et al., 2002; Guo et al., 2004). DOC also serves as a useful source of carbon and energy for heterotrophic microorganisms. The observed significant inverse correlation ($r = -0.816$, $n = 8$, $p<0.01$) between the concentrations of DOC and DO suggests the importance of DO in controlling the abundance of DOC due to activities of heterotrophic microorganisms. DOC concentrations are also influenced by the levels of nutrients. This was evident from the significant negative correlations of DOC with both nitrate ($r = -0.807$, $n = 8$, $p< 0.01$ in monsoon, and $r = -0.706$, $n = 9$, $p< 0.02$ in pre-monsoon) and silicate ($r = -0.725$, $n = 8$, $p< 0.02$ in monsoon, and $r = -0.604$, $n = 9$, $p<0.05$ in pre-monsoon). However, phosphate concentrations had a variable effect on the

levels of DOC. This contrasting correlations indicates different sources and control mechanisms for affecting DOC distribution.

4A.4.3 Distribution of TDCHO and HCl-TDCHO

Both marine and terrestrial organisms contain storage and structural carbohydrates. Structural compounds, such as chitin, cellulose and hemicellulose are relatively insoluble in seawater, and may be present as colloidal material or enzymatic degradation products in the operationally defined dissolved fraction in seawater. Water samples are generally hydrolyzed using dilute HCl (0.09 N) to estimate TDCHO (Burney and Sieburth, 1977; Parsons et al., 1984a). Dilute HCl-hydrolysis procedure for the estimation of TDCHO is not very effective for the hydrolysis of structural carbohydrates such as cellulose, hemicellulose and chitin (Pakulski and Benner, 1992, 1994). The use of dilute-HCl, therefore may underestimate the concentrations of dissolved carbohydrates in water samples. In view of this, Pakulski and Benner (1992) proposed the use of concentrated sulphuric acid for the complete hydrolysis of the structural polysaccharides to monosaccharides. The application of the method of Pakulski and Benner (1992, 1994) of pre-treatment (12 M H_2SO_4), and subsequent hydrolysis with dilute H_2SO_4 (1.2 M) results in yields of carbohydrates from natural samples that are equal to or greater than those obtained using dilute HCl method (Pakulski and Benner, 1994; Bhosle et al., 1998; D'Souza, 2004; Hayakawa, 2004). In the present study, when the method of Pakulski and Benner (1992, 1994) was applied to the water samples of the Mandovi estuary,

concentrations of TDCHO were 2 to 3 times greater than those obtained using dilute HCl hydrolysis method (Table 4A.1). This indicates that dissolved carbohydrates are composed of hydrolysable material and components that are resistant to HCl hydrolysis. Thus the application of these two methods for the analysis of dissolved carbohydrates in water samples provide useful information on the dynamics and the fate of various carbohydrate species such as TDCHO, HCl-TDCHO, MCHO, DPCHO, HCl-DPCHO and HR-DPCHO. Moreover, such an approach not only provides a better picture of carbohydrate distribution in estuarine waters, but also helps in understanding the role of these carbohydrate species in biogeochemical cycle of organic carbon.

The TDCHO values reported for the Mandovi estuary waters are similar to those reported earlier for other estuarine and coastal environments (Witter and Luther, 2002; Hung et al., 2003; 2005; Wang et al., 2006; Myklestad and Borsheim, 2007). For both the seasons, highest concentration of TDCHO was observed at station # 5. This is because the station # 5 receives water inputs from Asnoda and Dicholi rivers as well as from the Cumbarjua canal that connects Mandovi estuary with the Zuari estuary. TDCHO concentrations showed significant seasonal variation (Table 4A.2). This seasonal variability was influenced by the river run off, and organic matter production by phytoplankton and bacteria (Cherrier and Bauer, 2004).

Storage carbohydrates and low molecular weight polysaccharides are readily hydrolyzed in dilute HCl (0.09 N) (Burney and Sieburth, 1977; Pakulski and Benner, 1992, 1994; Hayakawa, 2004). Monosaccharides detected after

dilute HCl hydrolysis may therefore indicate the presence of storage polysaccharides or low molecular weight oligosaccharides in our samples. HCl-TDCHO varied spatially and seasonally, and accounted for greater proportion of TDCHO in pre-monsoon (~52%) than in monsoon (~37%). Such carbohydrates are of common occurrence in many diatoms and other microalgae. As compared to the monsoon season, Chl *a* concentrations were relatively greater during pre-monsoon season. Therefore, the elevated HCl-TDCHO fraction during the pre-monsoon season indicates contribution from freshly photosynthesized organic matter.

4A.4.4 Free monosaccharide (MCHO)

MCHO was an important component of TDCHO pool. MCHO concentrations showed significant spatial and seasonal differences (Table 4A.2). Low values in monsoon may be due to low phytoplankton production due to light limitation but not due to nutrient limitation. Low saline waters of the estuary, especially of station # 8 during the monsoon, and of the stations # 5 and # 8 during the pre-monsoon had higher concentration of MCHO. At these stations, Chl *a* concentrations were also high (Fig. 4A.3). During both the monsoon and pre-monsoon seasons, MCHO concentrations did not show significant correlations with DOC or with the carbohydrate species suggesting different sources and/or inputs. As compared to the monsoon season (12.6 ± 5.2%), MCHO contribution to the TDCHO was higher during the pre-monsoon season (32.7 ±9.42%). This implies higher production of MCHO by microorganisms and/or a net result of in situ hydrolysis of polysaccharides or

decomposition processes during the pre-monsoon season. Furthermore, it is suggested that the aggregation of DPCHO into particles could result in a higher proportion of MCHO in the water column (Engel et al., 2004). Low MCHO contribution to the TDCHO in the monsoon season was likely to be due to low rates of heterotrophic decomposition and/or utilization by heterotrophic populations.

MCHO was not the major (8 to 48% of TDCHO) constituent of TDCHO pool in the Mandovi estuary (Table 4A.1). Similar observations have been reported from coastal and oceanic waters (Bhosle et al. 1998; Hung et al., 2003). In contrast, for some temperate and Arctic rivers, TDCHO pool is mostly dominated by MCHO (80% of TDCHO) (Hung et al., 2001, 2005; Witter and Luther, 2002; Gueguen et al., 2006; Wang et al., 2006; Cai et al., 2008). Differences in the relative distribution of MCHO and DPCHO in the marine/ estuarine waters and the temperate and arctic rivers indicate different transformation pathways and mechanisms in TDCHO pool (Wilkinson et al., 1997; Hung et al., 2005). This is probably because in marine waters, sources of TDCHO are mostly from the in situ primary production by phytoplankton and bacterial metabolism with less decomposition and thus a higher DPCHO fraction (Pakulski and Benner, 1994). On the other hand, in river system, most TDCHO comes from allochthonous sources and undergoes intensive decomposition, and thus contain a lower DPCHO fraction (Table 4A.1) (Hedges et al., 1994; Hung et al., 2001, 2005; Gueguen et al., 2006; Cai et al., 2008).

4A.4.5 Dissolved polysaccharides DPCHO, HCl-DPCHO and HR-DPCHO

In the Mandovi estuary, DPCHO accounted for 76 to 92 % (average 87.4 ±5.2 %), and 52 to 79 % (average 67.3 ±9.4 %) of the TDCHO during the monsoon and pre-monsoon season, respectively suggesting that it was the predominant constituent within the TDCHO pool (Table 4A.1). This implies that TDCHOs of estuarine and marine waters are relatively rich in DPCHO (Bhosle et al., 1998, Pakulski and Benner, 1994, Hung et al., 2003; Wang et al., 2006).

Within the polysaccharide pool, HCl-DPCHO contributed from 14.7 to 38.9 % and from 12.7 to 64.6 % of DPCHO in the monsoon and pre-monsoon season, respectively (Table 4A.1). These polysaccharides indicate the presence of storage polysaccharides in our samples. Higher contribution of HCl-TDCHO to the DPCHO suggests production of these polysaccharides by phytoplankton during the pre-monsoon season.

The contribution of HR-DPCHO to the DPCHO and TDCHO were higher in the monsoon than in the pre-monsoon season (Table 4A.1). The monsoon season is characterized with greater precipitation and river run-off. River run-off is known to carry allochthonous organic matter derived from surface soils and decaying plant litters as well as polymers produced by microorganisms (Wilkinson et al., 1997). Thus, the HR-DPCHO input from the river was more important during the monsoon season. HR-DPCHO fraction consisted of structural polysaccharides and glycoproteins (Pakulski and Benner, 1992, 1994; Bhosle et al., 1998; Hayakawa 2004). Structural polysaccharides such as cellulose, hemicellulose, and pectins are abundant

in vascular plant tissues (Aspinall, 1970). Moreover, decomposition of plant litter would release polysaccharides, contributing to the higher percentage of HR-DPCHO during the monsoon season. Abundance of HR-DPCHO in TDCHO and DPCHO suggest that these compounds were resistant to biological degradation. The fate of these polysaccharides in fresh water and marine/estuarine waters appear to be different. In seawater samples, concentration of these polysaccharides increased with increasing water column depth suggesting that they were not preferentially utilized by heterotrophic organisms (Pakulski and Benner, 1992, 1994; Bhosle et al., 1998). Conversely, in the lake waters, HR-DPCHOs were less abundant than HCl-DPCHO suggesting that these compounds were utilized by the heterotrophic organisms (Hayakawa, 2004). These polysaccharides can be readily susceptible to enzymatic degradation in spite of being refractory to acid hydrolysis (Pakulski and Benner, 1994; Hayakawa, 2004). Additional data from different aquatic environments are needed in order to better understand the dynamics, fate and sources of these polysaccharides.

4A.4.6 MCHO/DPCHO ratio

During the monsoon, the ratio was low (0.08 to 0.3; average 0.14) while it was high (0.27 to 0.93; average 0.51) in the pre-monsoon season (Fig. 4A.6). The higher ratios indicate higher stability and biomass during pre-monsoon season. The higher ratios may not be explained by excretion from healthy cells (Myklestad, 1989), but rather a combination of cellular and in situ degradation (Myklestad et al., 1982; Ittekkot et al., 1981; Smith et al., 1992;

Meon and Kirchman, 2001). Ittekkot et al. (1981) reported increasing amounts of MCHO during the development of phytoplankton bloom, and the MCHO/DPCHO ratio reached ~ 1 at the late stages of the bloom. Small spatial differences in MCHO concentrations (<10 μM C) at many stations suggest production and utilization of MCHO by microorganisms in the surface waters of the estuary. DPCHO was consistently the dominant fraction of TDCHO pool but the ratio of both MCHO and DPCHO to TDCHO showed some spatial and seasonal differences. The observed differences in the MCHO/TDCHO and DPCHO/TDCHO ratio indicated transformation between carbohydrates and other organic carbon species at the sampling stations and the season. Increase in water temperature, microbial activity and photochemical reactions could be responsible for the observed transformation.

4A.4.6 Relationship between MCHO and TDCHO

During monsoon season, MCHO did not show significant correlations with TDCHO or with its fractions. Similarly, there were no correlations between MCHO and TDCHO either in the study of Harvey (1983) or in that of Burney et al. (1979). The lack of correlations between these parameters during monsoon suggests the presence of large quantities of newly biosynthesized carbohydrates that disturb the MCHO vs TDCHO relationship. In contrast, in pre-monsoon season, MCHO gave highly significant positive correlations with HCl-TDCHO ($r = 0.895$, $n = 9$, $p<0.001$) and HCl-DPCHO ($r = 0.895$, $n = 9$, $p<0.02$). However, it was surprising that free monosaccharides and

polysaccharides follow such similar pattern of release and uptake. It also seems interesting that MCHO as well as HCl-TDCHO and HCl-DPCHO were not used during their transport through the estuary. MCHO, in part can be derived from the degradation of DPCHO resulting in inverse relationship between these two parameters (Pakulski and Benner, 1994; Bhosle et al., 1998). However, no such inverse correlation was recorded in the present study.

4A.4.7 Carbohydrate yields

The contribution of carbohydrate-carbon to DOC is defined as carbohydrate yield. Carbohydrate yield is a useful tool to assess degradation state of organic matter. Yield is high for the freshly derived organic matter, and low for the degraded organic matter. Yields of most of the carbohydrate fractions were higher during the monsoon season and lower in pre-monsoon season (Fig. 4A.7). Higher yields in monsoon indicate the presence of carbohydrate rich dissolved organic matter probably derived from terrestrial sources. This implies transport of carbohydrates rich dissolved organic matter (DOM) from the river to the coastal waters during the monsoon season. Opsahl and Benner (1999) observed that carbohydrate yields rapidly decreased during the degradation of organic matter derived from mangrove leaves. Relatively low total carbohydrate yields (11 to 23 %) were recorded for the pre-monsoon season. These yields are in the range of values recorded for the freshly derived organic matter (15 to 30 %) from phytoplankton (Pakulski and Benner, 1994). Thus during both the seasons, carbohydrate rich dissolved

organic matter, probably derived from different sources was present in the Mandovi estuary. This carbohydrate rich dissolved organic matter can serve as a useful source of carbon and energy for the heterotrophic organisms.

4A.4.8 Monosaccharide composition of TDCHO

During the monsoon season, water samples from a few stations in the Mandovi estuary were analyzed for the monosaccharide composition of TDCHO. Examination of the monosaccharide composition of TDCHO provides information about the accumulation or preferential utilization of individual monosaccharides and the quality of the TDCHO. Glucose was most abundant fraction (37.7 to 50.6 %), followed by fucose (7.5 to 24 %), galactose (8.9 to 16.6), rhamnose (3.7 to 15.2 %), arabinose (2.7 to 18%), xylose (ND to 14 %), mannose (ND to 9.7%) and ribose (ND to 8%) in surface DOM (Fig. 4A.8). This monosaccharide composition of the TDCHO is similar to that described earlier for other estuarine and near shore environments (Borch and Kirchman, 1997; Biersmith and Benner, 1998; Meon and Kirchman, 2001). The dominant neutral sugar varies among cultured species and physiological state. Similarly, monosaccharide composition will be influenced by the contribution from terrestrial organic matter. Glucose abundance is not surprising as this sugar is most abundant in both terrestrial and marine phytoplankton. Fucose, rhamnose, galactose, glucose containing polymers are released by many diatom species. Fucose and rhamnose are reliable biomarkers of algal material. Similarly, plant hemicellulose polysaccharides contain relatively large amounts of xylose and arabinose in

the form of arabinoxylan, arabino glucuronoxylan etc (Moers et al., 1990). Moreover, rhamnose, arabinose and xylose are important constituents of dissolved organic matter produced during the decomposition of mangrove leaves (Bhaskar, unpublished results). This is in contrast to other riverine systems (e.g., Hedges et al., 1994; Benner and Opsahl, 2001; Repeta et al., 2002), where soils are expected to be the main source of DOM. Substantial contribution of xylose (11 to 14 mol %) and arabinose (2.7 to 18 mol %) to the TDCHO was observed for the waters of the Mandovi estuary. The Mandovi estuary has thick mangrove vegetation along its blank. Therefore, the observed spatial differences in the individual monosaccharide may also have been influenced by mangrove derived organic matter.

4A.4.9 Distribution and seasonal variation of dissolved uronic acids

Uronic acids are carboxylated acidic polysaccharides. They are surface active compounds, and although present as a minor constituent of total carbohydrates, they are actively involved in many environmental processes including production of humic acid, detoxification of toxic chemicals, removal of metal ions, adsorption of dissolved organic matter, production of macroaggregates and microbial adhesion (Yamaoka, 1983; Decho, 1990; Mopper et al., 1995; Leppard, 1997; Santschi et al., 1998; Jain and Bhosle, 2009).

Distribution, fate and cycling of uronic acids in estuarine and marine waters is not well known (Mopper, 1977; Bergamaschi et al., 1999; Hung et al., 2003, 2005; Khodse et al., 2007, 2008). In the Mandovi estuary, DURA

showed significant seasonal variations and varied from 2.3 to 10.8 μM C (Table 4A.1, 4A.2). These DURA concentrations are in the range of values earlier reported by Hung et al. (2001, 2005) for the Galveston Bay (1.0 to 8.3 μM C) and the Trinity river (5.2 to 25.7 μM C). The contribution of DURA to TDCHO was low in monsoon (~ 5.5 to 14.9 %) and high in pre-monsoon (~ 17.3 to 33.6 %). Using the spectrophotometeric method, Hung et al. (2001, 2005) reported that DURA accounted for ~4 to 17 % and 10 to 30 % of TDCHO in Galveston Bay and in Trinity river, respectively. The contribution of uronic acids to total carbohydrates varied from ~ 5 to 23 % in the suspended particles of the northern Indian Ocean (Khodse et al., 2007). Based on GC method uronic acids accounted for 9.89 % of the total carbohydrates of the sedimenting particles collected from the Dabob Bay (Bergmaschi et al., 1999).

The contribution DURA-C to DOC did not show seasonal variation during the monsoon (~2 to 5 %, average 3.25 ±0.98 %) and the pre-monsoon (~2 to 5 %, average 3.84 ±1.17 %) season. Uronic acid yields for the suspended particulate matter of northern Indian Ocean varied from 0.2 to 6.3 % (Khodse et al., 2007). The average uronic acids yield for the suspended particles collected from the Gulf of Mexico was 0.64 % and 0.70 % in 2000 and 2001, respectively (Hung et al., 2003). For the sedimenting particles of the Sagami Bay, Japan uronic acid yield was 0.7 % (Hamanaka et al., 2002). For the Trinity river, DURA yields varied from 1 to 5 % (Hung et al., 2005). DURA showed fairly good positive relationships with DOC during the monsoon ($r = 0.614$, $n = 8$, $p<0.1$) and the pre-monsoon season ($r = 0.664$, n

= 9, $p<0.05$). Similarly, DURA also showed positive relationships with TDCHO during the monsoon (r = 0.614, n = 8, $p< 0.1$) and the pre-monsoon (r = 0.869, n = 9, $p< 0.001$). These correlations indicate a common origin for these compounds.

Many microorganisms, vascular plants, and other organisms are known to produce uronic acids (Bergamaschi et al., 1999; Hung et al., 2003; Khodse et al., 2007, 2008). This implies that DURA concentration is not only controlled by phytoplankton production and bacterial assimilation or degradation but also regulated by other biological activities (Leppard 1997). Therefore additional inputs and selective preservation may be responsible for the variation in the distribution of DURA in the Mandovi estuary. Concentrations of metal ions in the growth medium are also known to influence the production of uronic acids by microorganisms. Adsorption by particles due to their surface active nature, microbial utilization and degradation are the other factors that may have influenced concentration of DURA in the Mandovi estuary.

4A.4.10 Factors influencing the distribution of carbohydrates and uronic acids

During the monsoon season, Chl *a* concentrations, total phytoplankton cell counts, diatom cell counts and dinoflagellate cell counts did not show significant correlations with TDCHO, its various fractions and DURA. Thus, during the monsoon season, carbohydrates concentration was not influenced by these factors and mostly by river run-off. In contrast, Chl *a* concentrations

showed significant positive correlations with the concentrations of MCHO ($r = 0.618$, $n = 9$, $p<0.05$), TDCHO ($r = 0.748$, $n = 9$, $p< 0.01$), HCl-TDCHO ($r = 0.744$, $n = 9$, $p<0.01$), DPCHO ($r = 0.610$, $n = 9$, $p<0.05$), HCl-DPCHO ($r = 0.741$, $n = 9$, $p<0.01$) and DURA ($r = 0.636$, $n = 9$, $p<0.05$). These correlations suggest that TDCHO, its fractions and DURA were mostly derived from phytoplankton during the pre-monsoon season.

Phytoplankton composition is another factor that influence the concentrations of TDCHO and DURA. In the Mandovi estuary, the phytoplankton composition changed with space and time. *Navicula maculosa.*, *Pleurosigma elongatum.*, *Rhizosolenia* sp., *Chaetoceros* sp., *Coscinodiscus* sp. and *Leptocylindrus minimus,* and several unidentified dinoflagellates were dominant during the monsoon season. Whereas, *Chaetoceros glandazii*, *Chaetoceros lascinious, Melosira* sp., *Coscinodiscus oceulus*, *Fragillaria oceanica*, *Pluerosima strigosum*, *Rhizosolenia* sp. *Skeletonema costatum*, and *Thalassiothrix fraufeldii*, were dominant during the pre-monsoon season (Prabhu Matondkar, personal communications). The observed changes in phytoplankton composition may be responsible for the observed variations in the concentrations of carbohydrate species in the Mandovi estuary.

With the exception of a few stations, diatoms were abundant in the Mandovi estuary. Diatoms are known to produce large amounts of carbohydrates and uronic acids in culture (Handa, 1969; Myklestad, 1989; Bhosle et al 1998; Meon and Kirchman, 2001), and in natural waters (Ittekkot et al., 1981; Tanoue and Handa, 1987; Bhosle and Wagh, 1989; Khodse et

al., 2007). Laboratory grown diatom culture, bacteria and natural phytoplankton populations are well known to produce lower amounts of carbohydrates during early logarithmic growth, and higher amounts of carbohydrates during stationary growth phase (Myklestad, 1977; Hitchcock, 1978; D'Souza and Bhosle, 2001). It is also observed that DURA production by diatoms is higher in the stationary growth phase and lower during the early logarithmic phase of growth (Khodse and Bhosle, unpublished results). Therefore, the observed variations in concentrations and yield of carbohydrates and uronic acids may reflect the effect of the growth phase of microorganisms.

Microbial activity is yet another factor that may influence carbohydrates concentrations in the estuarine waters. Microbial activity increases with increasing water temperature, preferentially utilizing specific and more labile DOC compounds (Wilen, 2000; Apple et al., 2006). In the Mandovi estuary, bacterial cell numbers showed a significant negative correlation with TDCHO:DOC ratio suggesting preferential microbial degradation of carbohydrates (Fig. 4A.9a). Microbial degradation enhanced with the increase in situ water temperature was also supported by the significant negative correlation between the TDCHO:DOC ratio and the in situ water temperature (Fig. 4A.9b). The decrease in dissolved carbohydrate concentrations from winter to spring was ascribed to microbial activity (Sweet and Perdue, 1982). In the Mandovi estuary, both bacterial abundance and productivity were influenced by water temperature with higher bacterial abundance and production in pre-monsoon season. Higher temperature

resulted in higher bacterial activity was evident from significant positive relationship between bacterial production and temperature, especially during the pre-monsoon season ($r = 0.788$, $n = 9$, $p<0.01$). Bacterial abundance and production were higher during the summer and showed positive correlations with in situ water temperature (Findlay et al., 1991; Apple et al., 2006).

During monsoon season, temperature showed negative correlation with TDCHO ($r = -0.721$, $n = 8$, $p<0.02$), HCl-TDCHO ($r = -0.549$, $n = 8$, $p<0.02$), DPCHO ($r = -0.757$, $n = 8$, $p<0.01$), and HCl-DPCHO ($r = -0.676$, $n = 8$, $p<0.1$). From these correlations, it was evident that during monsoon season, carbohydrate concentrations decreased in response to in situ water temperature dependent uptake and degradation reactions by bacteria and photochemical processes, leaving behind more refractory compounds. This argument is well supported by the high abundance of HR-DPCHO in the waters of the Mandovi estuary during the monsoon season. Therefore, during monsoon, organic matter enriched in relatively less degradable carbohydrate compounds such as HR-DPCHO will be transported from the Mandovi river into the ocean.

Carbohydrate and uronic acid concentrations are a function of rates of production and degradation processes. DURA appear to be relatively less degradable than the TDCHO, was evident from the significant positive correlation between water temperature and DURA:TDCHO ratio (Fig. 4A.9c). Similarly, DURA are also more resistant to degradation than the HR-DPCHO. This was also apparent from the significant positive relationship between DURA:HR-DPCHO ratio and the in situ water temperature (Fig. 4A.9d). The

positive intercept suggests that some extra DURA compounds were produced in situ at elevated temperatures or that they are more refractory than more labile bulk TDCHO compounds. This is supported by the significant negative correlation between TDCHO/DOC and temperature. Thus, this positive correlation between DURA/TDCHO and temperature more likely indicates that DURA compounds are more stable compounds than the carbohydrates. This was also evident from the abundance of DURA in water, suspended and sedimenting particles and marine sediments (Bergamaschi et al., 1999; Hung et al., 2001, 2003; Khodse et al., 2007, 2008).

Effect of salinity on MCHO, TDCHO and DURA was variable. In the monsoon season, salinity did not show significant correlations with MCHO, TDCHO species, and DURA. Conversely, salinity showed significant inverse correlations with TDCHO ($r = -0.643$, $n = 9$; $p<0.05$) and DURA ($r = -0.626$, $n = 9$, $p<0.05$) during the pre-monsoon season. Our results suggest that the concentrations of TDCHO and DURA were widely variable in the estuary on a short time-scale. These data also suggest that a part of the TDCHO and DURA was coming from the river and was conservative in the pre-monsoon season in this estuary. To the best of our knowledge, this is the first report suggesting the conservative behaviour of DURA.

TDCHO and DURA production by microorganisms is also influenced by the levels of nutrients, especially nitrate, phosphate and silicates in growth medium, and in natural waters (Myklestad 1989; D'Souza and Bhosle, 2001). Many microorganisms such as diatoms and bacteria generally produce higher amounts of carbohydrates under nutrient deficient or depleted conditions.

Concentrations of nitrate, phosphate and silicate varied spatially and were higher in the monsoon and lower in pre-monsoon season. We also observed negative correlations between concentrations of TDCHO and the concentrations of nutrients, especially phosphate ($r = -0.549$, $n = 9$, $p>0.05$), nitrate ($r = -0.465$, $n = 9$, $p>0.05$) and silicate ($r = -0.747$, $n = 9$, $p<0.01$) during the pre-monsoon season. It appears that under nutrient limited condition, uronic acids production by microorganisms is induced (Decho 1990; Khodse and Bhosle, 2010). Similarly, DURA production was influenced by phosphate ($r = -0.514$, $n = 9$, $p>0.05$), nitrate ($r = -0.689$, $n = 9$, $p<0.02$) and silicate ($r = -0.670$; $n = 9$, $p<0.05$). Therefore, the variable nutrient concentrations at these locations have influenced the production of carbohydrates and DURA by microorganisms in the Mandovi estuary.

4A.5 Conclusions

Concentrations of carbohydrates and uronic acids showed strong seasonal and spatial variations. TDCHO concentrations and yields were higher during the monsoon season. Polysaccharides, especially HR-DPCHO were the major constituents of TDCHO. TDCHO:DOC ratio showed a significant negative correlation with bacterial cell numbers and water temperature suggesting carbohydrates concentration was controlled by bacterial number as well as temperature. It appears that DURAs were relatively more resistant to degradation as compared to the TDCHO. Many physical, biological and chemical factors and freshwater run-off appear to influence the distribution and fate of TDCHO and DURA in the Mandovi estuary.

Chapter 4B

Distribution, nature and sources of suspended particulate organic matter in the Mandovi estuary, west coast of India

4B.1. Introduction

Estuaries are sites of exchanges between terrestrial and oceanic organic matter (OM), and also are active zones where dissolved and particulate materials are produced, transformed, or removed by physical and biological processes. In estuaries, organic matter is derived from allochthonous (terrestrial plants and soils), and autochthonous (in situ aquatic production) sources (Degens, 1982). Estuarine environments are characterized by high levels of biological production, and are also active sites of heterotrophic metabolism (Bianchi, 2007). These biogeochemical transformations require chemical energy in the form of labile organic matter. Therefore a fundamental issue in estuarine science is to understand the origin, abundance and reactivity of organic matter.

The bulk parameters such as stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes have been employed to distinguish between autochthonous and allochthonous sources of carbon (Cifuentes et al., 1996; Goni et al., 2003; Malet et al., 2008). Carbon isotope ratio is used to distinguish $\delta^{13}C$ depleted terrestrial material from the more $\delta^{13}C$ enriched marine OM (Saliot et al., 2002; Malet et al., 2008).

Nitrogen isotopes also have been used to distinguish between natural and anthropogenic sources of nitrate. $\delta^{15}N$ ranges, however, are not rigorously invariable because isotopic fractionation can occur within both ground water and soil. Isotopic fractionation is significantly influenced by denitrification within soil and ground water, the volatile loss of ammonia from manure, and the uptake of nitrate by microbes or algae; this complicates efforts to trace nitrogen sources from $\delta^{15}N$ values. Despite

the complexity of isotopic fractionation during nitrogen cycle, numerous studies have demonstrated that nitrogen isotopes of organic matter do, at least in part, reflect their source (Sigleo and Macko, 2002; Thomas et al., 2002).

The ratios of POC/TPN and POC/Chl *a* also have been employed to assess the nature and sources of OM. The POC/TPN ratio of terrestrial organic matter vary over a wide range (12-400) and decrease during diagenesis, whereas, the POC/TPN ratios of phytoplankton are less variable (eg. 6-8; Ertel et al., 1986; Hedges et al., 1986; Onstad et al., 2000; Jennerjahn et al., 2004). Similarly, POC/Chl *a* ratio has been used to assess the presence of freshly derived algal material. POC/Chl *a* ratio is low (20 to 30 w/w) in freshly grown algal material and increase with degradation.

Carbohydrates are the major organic compounds produced by autotrophic organisms during the process of photosynthesis, and serve as common structural and storage components in both marine and terrestrial plants. Carbohydrates may account 2-39 % of POC of the suspended (D'Souza and Bhosle, 2001; Khodse et al., 2007) and sedimenting particles (Hernes et al., 1996; D'Souza et al., 2003; Panagiotopoulos and Sampere 2005), and 3 to 26% in marine sediments (Cowie and Hedges, 1994; Burdige et al., 2000; Jensen et al., 2005; Khodse et al., 2008). They serve as an important energy source for various heterotrophic organisms in the water column and sediments (Decho, 1990). During the transport of organic matter from the euphotic zone to greater depths storage carbohydrates are preferentially utilized by in situ organisms (Ittekkot et

distinguish between terrestrial and marine sources, with high ratios (>20) indicating a terrestrial source. However, high ribose content in a sample may indicate contribution from bacteria and calcareous nanoplankton (Cowie and Hedges, 1984). Ittekkot et al. (1984) proposed the use of the arabinose/fucose ratio to identify the inputs from calcareous and siliceous material in sediment trap samples. Similarly, increase in deoxysugars (mol%) (rhamnose plus fucose) associated with a concomitant decrease in glucose (mol%) indicates the presence of degraded organic matter in both marine and terrestrial samples (Hamilton and Hedges, 1988). Moreover, contribution of neutral carbohydrate carbon to organic carbon defined as yield provides information on the degradation state of organic matter, where high yield typically indicates fresh OM (Cowie and Hedges, 1984; Skoog and Benner, 1997).

Many organisms are also known to produce acidic carbohydrate such as uronic acid. Production of uronic acid is influenced by season, growth phase, levels of nutrient and metal ions (Whistler and Richards, 1970; Bergamaschi et al., 1999). Total particulate uronic acid (TPURA) may account for ~ 5 to 23 % of total particulate carbohydrates (TPCHO), and 0.2 to 6.3 % of POC in aquatic environments (Hung et al., 2003; Khodse et al., 2007). Although present as a minor component of organic carbon, these acidic carbohydrates are involved in numerous biogeochemical processes described in chapter 2.

Tropical estuaries of the west coast of India are strongly influenced by south west (SW) monsoon (June to September). Due to heavy river run off, the Mandovi estuary behaves like a fresh water system during SW

monsoon. In contrast, during the pre-monsoon season (October to May) the estuary acts as a marine system extending from the mouth of the estuary to several kilometers upstream. The effect of monsoon and non-monsoon seasons on the physical, chemical and biological characteristics of the estuary has been well characterized (Qasim and Sen Gupta, 1981; De Sousa, 1983; Devassy and Goes, 1988; Ansari and Parulekar, 1993; Shetye et al., 1995; Ram et al., 2003; Shetye et al., 2007; Gonsalves et al., 2009; Partihary et al., 2009; Kessarkar et al., 2010). Conversely, little is known, on molecular level, concerning the biochemical characteristics of OM in this tropical monsoon driven estuary. Such studies on detailed molecular composition, nature, sources and spatial and temporal variation would help in understanding the dynamics, origin and nutritional value of the OM (Hedges et al., 1994; Davies et al., 2009; Kaiser and Benner, 2009).

Distribution and cycling of dissolved carbohydrates and uronic acids has been described in the earlier chapter. In this chapter, the distribution and cycling of particulate carbohydrates are evaluated. In view of this, the aims of the present study were to: 1) study the distribution of total particulate carbohydrates (TPCHO), total neutral particulate carbohydrates (TNPCHO), and total particulate uronic acids (TPURA), 2) assess the nature and sources of OM, and 3) to identify the effect of monsoon on the distribution, nature and sources of particulate organic matter (POM).

4B.2 Materials and methods

4B.2.1 Sampling and analytical methods

As described in chapter 4A, surface (~1 m) water samples were collected using 5 liter Niskin water samplers from 9 locations in the Mandovi estuary during the monsoon (August 2005) and pre-monsoon (March 2007) seasons (Fig. 4A.1). Water samples were collected for the analysis of temperature, salinity, nutrients (nitrate, phosphate, silicate), dissolved oxygen (DO), Chl *a* , phytoplankton cell counts, and total bacterial count (TBC). Immediately after collection, water was passed through 200 µm mesh nylon screen to remove large zooplankton, and then known aliquots were filtered through pre-combusted (450 °C, 4h) 47 mm GF/F filter papers (0.7 µm, Whatman) for the measurements of suspended particulate matter (SPM), POC, total particulate nitrogen (TPN), $\delta^{13}C_{oc}$ and $\delta^{15}N$, TPCHO, TNPCHO and TPURA. The filters were immediately transferred to clean screw capped scintillation vials and transported to the laboratory in an ice box. All samples were stored at - 20 °C prior to analysis. As and when required, data on temperature, salinity, nutrients, DO, Chl *a*, phytoplankton cell counts, and TBC were taken from chapters 4A.

4B.2.2 Determination of SPM, POC, TPN, $\partial^{13}C_{oc}$, and $\partial^{15}N$

GF/F filter containing particulate matter was washed with distilled water to remove salt and the filter was dried at 40 °C for 24 h. Filter was cooled and weighed on a microbalance to obtain the weight of SPM. POC was analyzed spectrophotometrically by the wet oxidation method (Parsons et al., 1984). Glucose was used as a standard. Analytical variation of the

POC was 4.9%. TPN was estimated following the method of Raimbault and Gerd (1991). EDTA was use as a standard. Analytical variation of method was 8.8% (Khodse et al., 2009).

In order to analyse $\delta^{13}C_{oc,}$ and $\delta^{15}N$, GF/F filters containing particulate material were treated with vapors of concentrated HCl to remove carbonates and oven dried at 40 °C for 24 h (Hedges and Stern, 1984). The bulk abundances of $\delta^{13}C_{oc,}$ and $\delta^{15}N$ in suspended matter on the GF/F filters were determined using an elemental analyzer (EA, Thermo Electron Model-112) coupled online via a conflo interface with an isotope ratio mass spectrometer (EA-IRMS< Delta Plus, Thermo) . Isotope ratios are reported in standard (∂) notation and expressed as per mil (‰) differences from a standard reference material as given below:

$$\delta X\ (‰) = [(R_{sample}/R_{standard})-1] \times 1000$$

Where X is the $^{13}C_{oc}$ or, ^{15}N, R is the corresponding ratio of $^{13}C/^{12}C$, or $^{15}N/^{14}N$, in a sample or standard (PDB for carbon and atmospheric N_2 for nitrogen). Precision of $\delta^{13}C_{oc}$ and $\delta^{15}N$ measurement was ±0.04 $^{0}/_{00}$ and ± 0.1‰, respectively based on replicate analysis of IAEA (International Atomic Energy Agency) standard.

4B.2.3 Determination of TPCHO, TNPCHO and TPURA

TPCHO was determined by phenol sulfuric acid method described in chapter 3B. TNPCHOs were analyzed by capillary gas chromatography method described earlier in chapter 3B. TPURA was determined following the method described in chapter 3B.

4B.2.4 Statistical analysis

As described in chapter 4A one-way ANOVA was carried out to understand seasonal variations in bulk parameters. Principal component analysis (PCA) was performed on a personal computer using the statistical software package version 5.0 (Statsoft, Inc.). The data matrix used for PCA consisted of POC/TPN ratio, POC/Chl *a* ratio, SPM content (mgl^{-1}), TBC count (cells l^{-1}), and concentrations (μgl^{-1}) of Chl *a*, TPCHO, TPURA, and monosaccharides (as mol%). The raw data matrix was normalized in order to nullify the influence of the components, which were present in higher concentrations. Normalization was carried out by taking the logarithm of each component value (as concentration or mol%) plus 1, i.e. log (x+1) where x was the value of each component. Factors were extracted after varimax rotation and when the eigenvalues were over 1.

4B.3 Results

4B.3.1 Distribution of bulk parameters

Range and average values of surface temperature, salinity, dissolved oxygen, phosphate, nitrate, silicate, Chl *a*, phytoplankton count and bacterial abundance are presented in Table 4B.1. Detailed descriptions of these parameters are presented in chapter 4A. During the monsoon season salinity was low due to very high freshwater discharge (60.3 Mcum/day, to 1809 Mcum/month) (Fig. 4B.1a). In contrast, in the pre-monsoon water discharge was very low resulting in salinity gradient which was influenced by tidal mixing (Fig. 4B.1a).

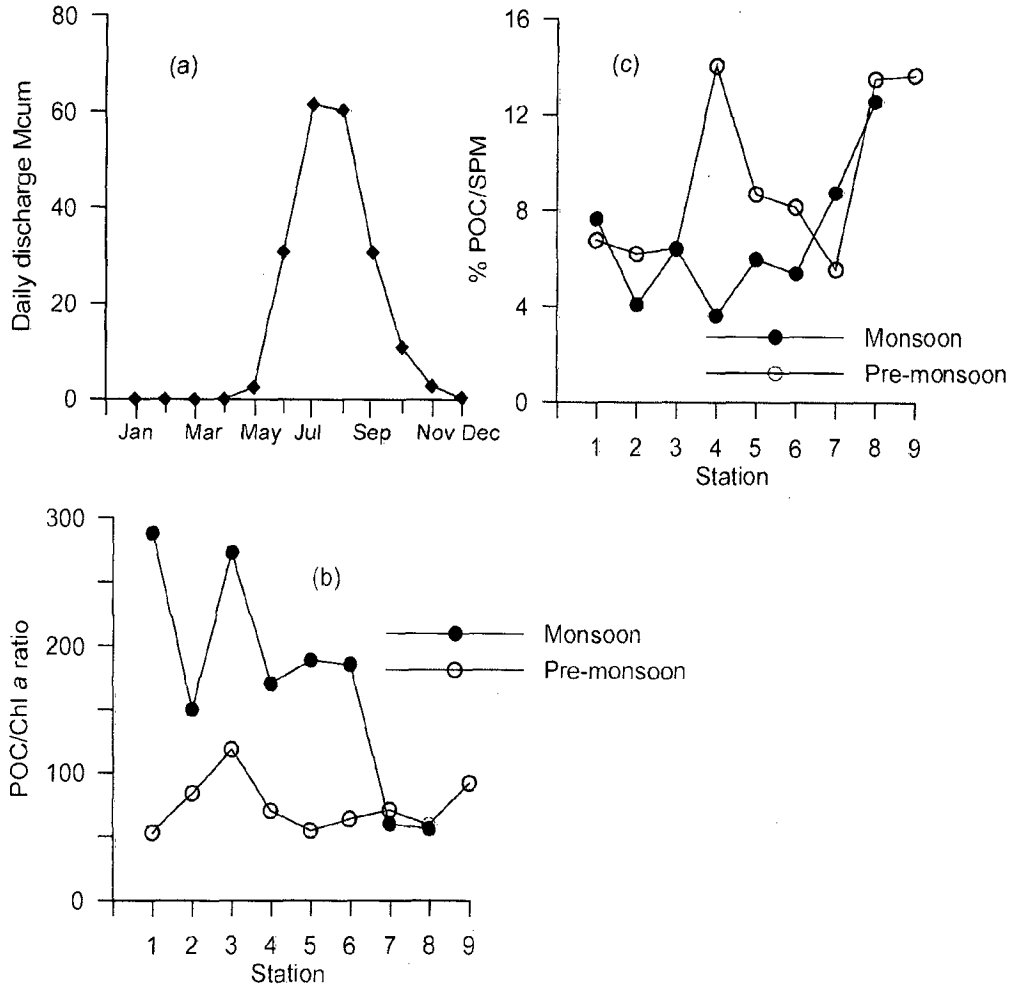


Fig. 4B.1. Variation of water discharge (a) and changes in POC/Chl *a* ratio (b), and %POC/SPM (c) in the Mandovi estuary. Water discharge data obtained from climatology of river runoff (average of from 1981 -1998).

4B.3.2 SPM, POC, TPN, POC/TPN, and $\delta^{13}C$ and $\delta^{15}N$

SPM varied from 2.92 to 18.42 mg l^{-1} and from 7.22 to 14.07 mg l^{-1} during the monsoon and the pre-monsoon season, respectively (Fig. 4A.2a).

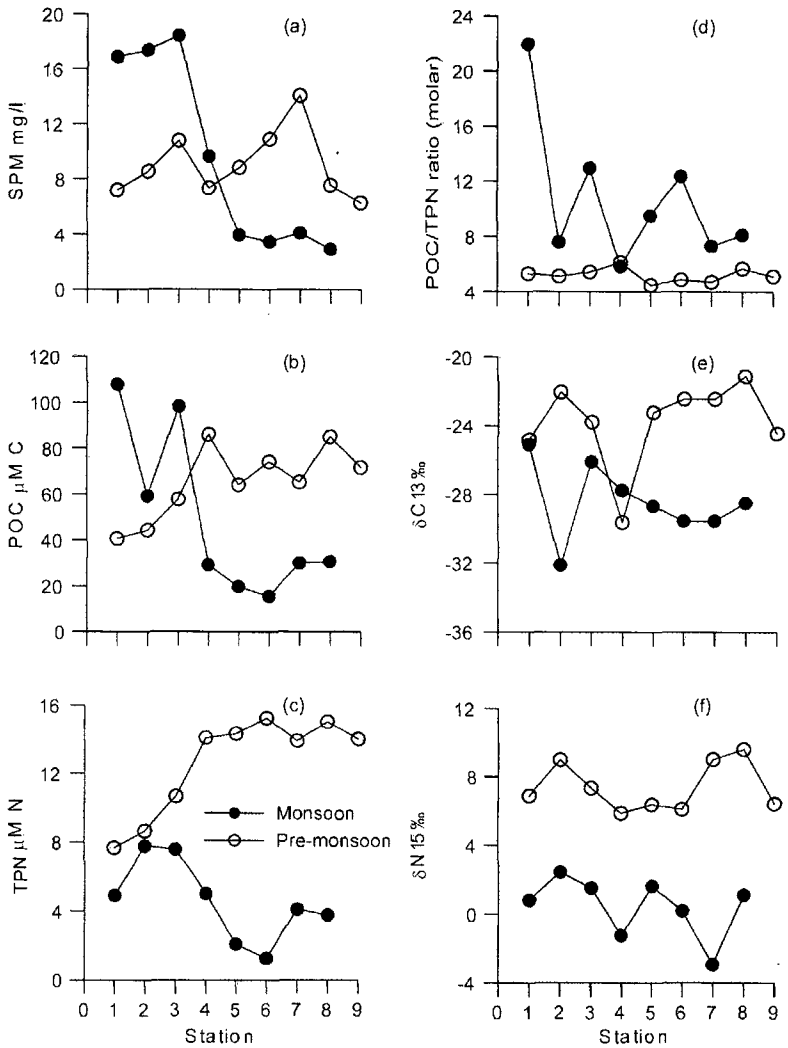


Fig. 4B.2. Concentration of SPM (a), POC (b), TPN (c), POC/TPN ratio (d), $\delta^{13}C$ (e) and $\delta^{15}N$ (f) in surface water of the Mandovi estuary during monsoon and pre-monsoon period.

SPM generally increased from the upstream region to the mouth of the estuary during the monsoon season and did not show any particular trend during the pre-monsoon season. During the monsoon season POC concentrations varied from ~16 to 108 µM C, and were higher at station # 1, # 2 and # 3 near the mouth of the estuary (Fig. 4B.2b). For the pre-monsoon season, POC ranged between ~ 41 and 86 µM C (Fig. 4B.2b). POC concentrations were low at stations # 1 and # 2 near the mouth and

relatively high at the upstream stations # 8 and 9. During both the seasons, TPN distribution in the estuary closely resembled to that of POC. (Fig. 4B.2c).

POC/TPN ratio was higher (3.8 to 22) during the monsoon and lower (4.5 to 6.1) in the pre-monsoon season (Fig. 4B.2d). POC/Chl *a* ratio varied from 56 to 288, and generally exhibited a decreasing trend from the mouth of the estuary to the upstream region of the estuary during the monsoon season (Fig. 4B.1b). For the pre-monsoon season, the ratio was low and varied from 53 to 118 (Fig. 4B.1b). The values of $\delta^{13}C$ and $\delta^{15}N$ varied from - 29.5 to -25.1 $^{o}/_{oo}$ and - 2.9 to 2.4 $^{o}/_{oo}$, respectively during the monsoon period (Fig. 4B.2e). During the pre-monsoon season, $\delta^{13}C$ and $\delta^{15}N$ ranged from - 29.6 to – 21.1 $^{o}/_{oo}$, and from 5.9 and 9.6 $^{o}/_{oo}$, respectively (Fig. 4B.2e).

4B.3.3 Distribution of TPCHO and TPURA

TPCHO showed strong spatial and seasonal variations, and varied from 2.35 to 9.30 µM C and 4.32 to 10.20 µM C during the monsoon and pre-monsoon seasons, respectively (Table 4B.1 and Table 4B.2). TPCHO accounted for 8.50 to 21.20 and 7.40 to 14.80% of POC during the monsoon and pre-monsoon season, respectively (Table 4B.2). The concentrations of TPURA ranged between 0.14 and 0.39 µM C during monsoon season and from 0.34 and 0.90 in the pre-monsoon season and accounted for 0.24 to 1.42 and 0.52 to 1.21 of POC, respectively (Table 4B.2). TPCHO/TPURA ratio showed spatial differences, and was relatively greater during the pre-monsoon season (Table 4B.2).

Table 4B.1 Minimum and maximum range, average, SD and *p* value (one way ANOVA) of various parameters during monsoon and pre-monsoon period.

	Monsoon			Pre-monsoon			ANOVA	Significance
Bulk parameters	Range	Average	SD	Range	Average	SD	*p*- value	
Temperature °C	27.4 - 28.3	27.7	±0.3	30.7 - 31.8	31.2	±0.4	1.10E-08	*P*<0.001
Salinity	0.1 -16.3	5.7	±7.5	17.5 - 34.6	28.6	±6.2	2.00E-06	*P*<0.001
DO mgl^{-1}	3.3 - 5.8	4.8	±1	2.9 - 4.3	3.5	±0.5	1.00E-03	*P*<0.001
NO_3 µM	3.7 - 8.9	7.1	±2.1	1.2 - 4.1	2.5	±1.1	9.80E-05	*P*<0.001
PO_4 µM	0.4 - 1.0	0.6	±0.2	0.4 - 0.8	0.6	±0.2	6.15E-01	*P*> 0.05
Si µM	43.9 - 126.9	91.9	±35.4	1.2 - 7.0	3.9	±2.1	6.20E-06	*P*<0.001
Chl *a* μgl^{-1}	1.0 - 6.5	3.8	±2.1	5.9 - 17.2	11.3	±3.9	3.30E-04	*P*<0.001
Phyto ($x10^4$ cells l^{-1})	0.3 - 3.8	1.0	±1.3	0.3 - 3.2	1.4	±1.1	6.60E-01	*P*> 0.05
TBC ($x10^8$ cells l^{-1})	6.0 - 29.1	12.8	±10.0	17.1 - 42.1	27.3	±9.4	1.50E-02	*P*<0.05
SPM mgl^{-1}	2.9 - 18.4	9.6	±6.9	6.3 - 14.1	9.1	±2.5	9.43E-01	*P*> 0.05
POC µM C	15.5 - 107.8	48.8	±36	40.6 - 86.0	65.4	±16	2.76E-01	*P*> 0.05
TPN µM N	2.1 - 7.8	4.9	±1.9	7.7 - 15.3	12.6	±2.9	3.00E-05	*P*< 0.001
POC/TPN at	3.8 -22.0	9.6	±5.2	4.5 - 5.7	5.2	±0.5	3.50E-02	*P*<0.05
$\delta^{13}C$ ‰	-32.1 to -25.1	-28.4	±2.2	-29.6 to -21.1	-23.8	±2.5	1.00E-03	*P*<0.001
$\delta^{15}N$ ‰	-2.9 to -2.4	0.4	±1.8	5.9 - 9.6	7.4	±1.4	5.00E-07	*P*<0.001
TPCHO µM C	2.4 - 9.3	5.8	±2.7	4.3 - 10.2	7.0	±2.3	3.55E-01	*P*> 0.05
TNPCHO µM C	2.1 - 6.7	3.8	±1.5	3.5 - 9.5	5.1	±2.2	1.31E-01	*P*> 0.05
TPURA µM C	0.14 - 0.39	0.27	±0.09	0.34 - 0.90	0.59	±0.18	7.90E-04	*P*<0.001
TPCHO-C %	8.5 - 21.2	13.9	±4.5	7.4 - 14.8	10.8	±2.2	1.27E-01	*P*> 0.05
TNPCHO-C %	4.6 - 14.0	9.7	±3.4	5.1 - 11.1	7.83	±2.2	3.09E-01	*P*> 0.05
TPURA-C %	0.24 - 1.42	0.7	±0.45	0.52 - 1.21	0.83	±0.25	5.12E-01	*P*> 0.05

nd = no data; DO = dissolved oxygen; NO3 = nitrate; PO4 = phosphate; Si = silicate; Chl *a* = chlorophyll *a*; Phyto = total phytoplankton; TBC = total bacterial count; BP = bacterial production; SPM = suspended particulate matter; POC = particulate organic carbon; TPN = total particulate nitrogen; $\delta^{13}C$ = carbon isotope; $\delta^{15}N$ = nitrogen isotope; TPCHO = total particulate carbohydrate; TNPCHO = total neutral particulate carbohydrate; TPURA = total particulate uronic acids

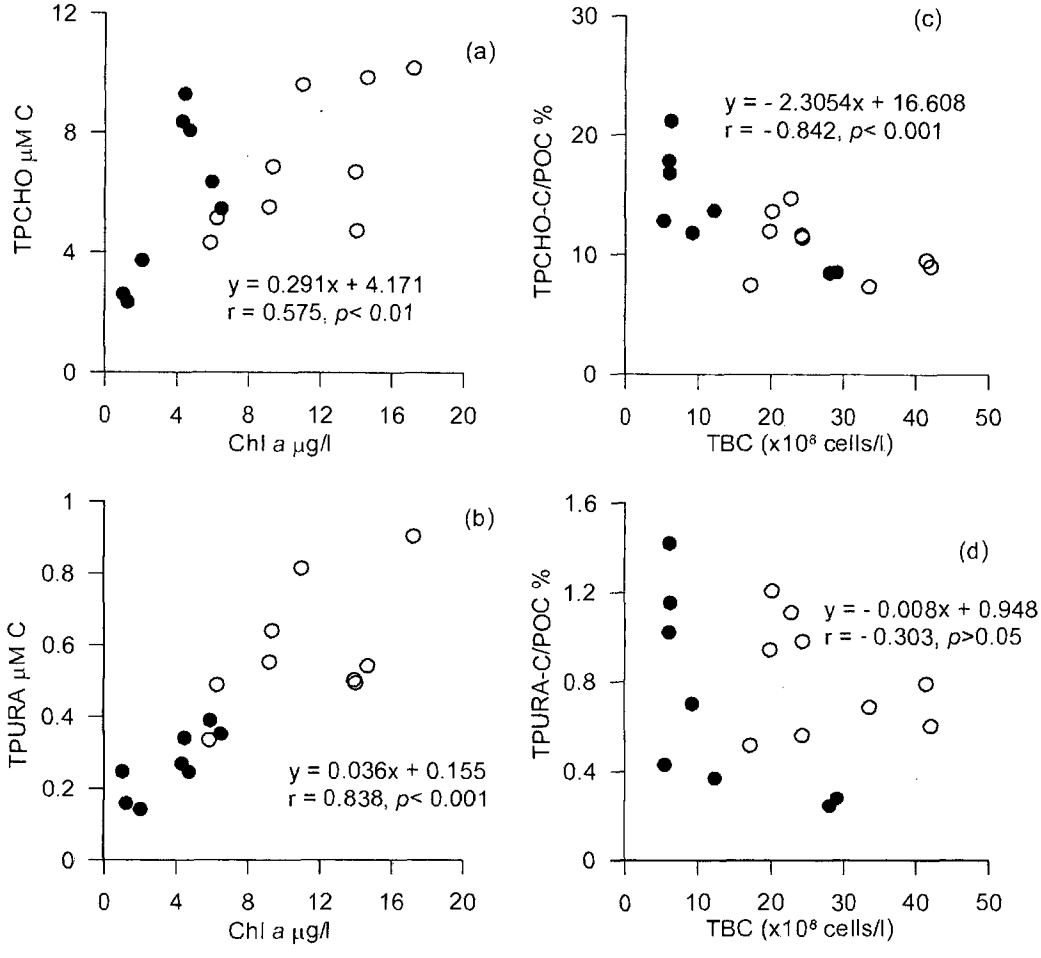


Fig. 4B.3 Relationships of Chl *a* with TPCHO (a), and TPURA (b); and of TBC with TPCHO-C/POC (c) and TPURA-C/POC (d) in surface water SPM of the Mandovi estuary. Data indicates monsoon (dark circle) and pre-monsoon (open circle).

Table 4B.2. Distribution of total particulate carbohydrates (TPCHO); total neutral particulate carbohydrate (TNPCHO), and total particulate uronic acids (TPURA) in surface water of the Mandovi estuary.

Station	TPCHO		TNPCHO			TPURA		
	a	b	a	b	c	a	b	c
Monsoon								
1	9.30 ±1.08	8.6	6.74 ±0.63	6.3	72.5	0.34 ±0.05	0.3	3.7
2	8.09 ±0.03	13.7	3.36 ±0.20	5.7	41.5	0.25 ±0.04	0.4	3.1
3	8.37 ±1.43	8.5	4.56 ±0.33	4.6	54.4	0.27 ±0.02	0.2	3.2
4	3.74 ±0.24	12.9	3.17 ±0.47	10.9	84.8	0.14 ±0.01	0.4	3.8
5	2.35 ±0.33	11.9	2.09 ±0.20	10.6	89.1	0.16 ±0.02	0.7	6.7
6	2.61 ±0.26	16.8	2.06 ±0.14	13.2	78.7	0.25 ±0.02	1.4	9.5
7	6.36 ±0.24	21.2	4.18 ±0.14	14.0	65.8	0.39 ±0.05	1.2	6.1
8	5.47 ±0.14	17.9	3.87 ±0.12	12.6	70.8	0.35 ±0.03	1.0	6.4
Pre-monsoon								
1	5.54 ±0.28	13.7	3.48 ±0.14	8.6	62.8	0.55 ±0.06	1.2	10.0
2	5.15 ±0.25	11.7	4.20 ±0.21	9.5	81.5	0.49 ±0.07	1.0	9.5
3	4.32 ±0.25	7.5	4.03 ±0.30	7.0	93.3	0.34 ±0.05	0.5	7.8
4	9.88 ±0.78	11.5	7.99 ±0.81	9.3	80.9	0.54 ±0.09	0.6	5.5
5	4.74 ±0.63	7.4	3.45 ±0.48	5.4	72.8	0.50 ±0.08	0.7	10.5
6	6.72 ±0.04	9.1	3.82 ±0.40	5.1	56.8	0.50 ±0.01	0.6	7.5
7	9.63 ±0.27	14.8	6.01 ±0.19	9.2	62.4	0.82 ±0.07	1.1	8.5
8	10.20 ±0.97	12.0	9.45 ±0.38	11.1	92.6	0.90 ±0.01	0.9	8.9
9	6.87 ±0.89	9.6	4.03 ±0.46	5.3	55.1	0.64 ±0.05	0.8	9.3

a = concentration μM C; b = % of POC; c = % of TPCHO; ± = represent standard deviation between replicates

Chl *a* concentrations of the SPM showed significant positive relationships with TPCHO and TPURA (Fig. 4B.3a, b). Bacterial abundance influenced TPCHO and TPURA yields. TBC showed negative correlation with TPCHO-C and TPURA-C to POC (Fig. 4B.3c, d).

4B.3.4 TNPCHO concentration and composition of the SPM

TNPCHO varied from 2.06 to 6.74 and 3.45 to 9.45 μM C and their contribution to POC ranged between 4.60 and 14.00% and from 5.1 and 11.1% during the monsoon and pre-monsoon seasons, respectively (Table 4B.2). TNPCHO accounted for 41.5 to 93.3% of TPCHO (Table 4B.2). TNPCHO yield was higher during the monsoon than pre-monsoon season (Table 4B.2).

The capillary gas chromatographic analysis revealed the presence of arabinose, fucose galactose, glucose, mannose, rhamnose, ribose, and xylose in the hydrolyzate of the SPM of the Mandovi estuary. Glucose (30 to 77%) was the most abundant monosaccharide followed by rhamnose (5 to 30%), galactose (9 to 25%), fucose (2 to 13%), mannose (4 to 11%), xylose (1 to 9%), arabinose (1 to 14%) and ribose (1 to 3%) during sampling period (Fig. 4B.4). Of these, glucose was most abundant monosaccharide accounting for 30 to 59 mol% and 60 to 77 mol% of the total TNPCHO during the monsoon and pre-monsoon season, respectively. Rhamnose, fucose, ribose, arabinose, xylose, and mannose were relatively more abundant in the monsoon than in pre-monsoon season (Fig. 4B.4). In contrast, glucose was the most abundant monosaccharide during the pre-monsoon (Fig. 4B.4).

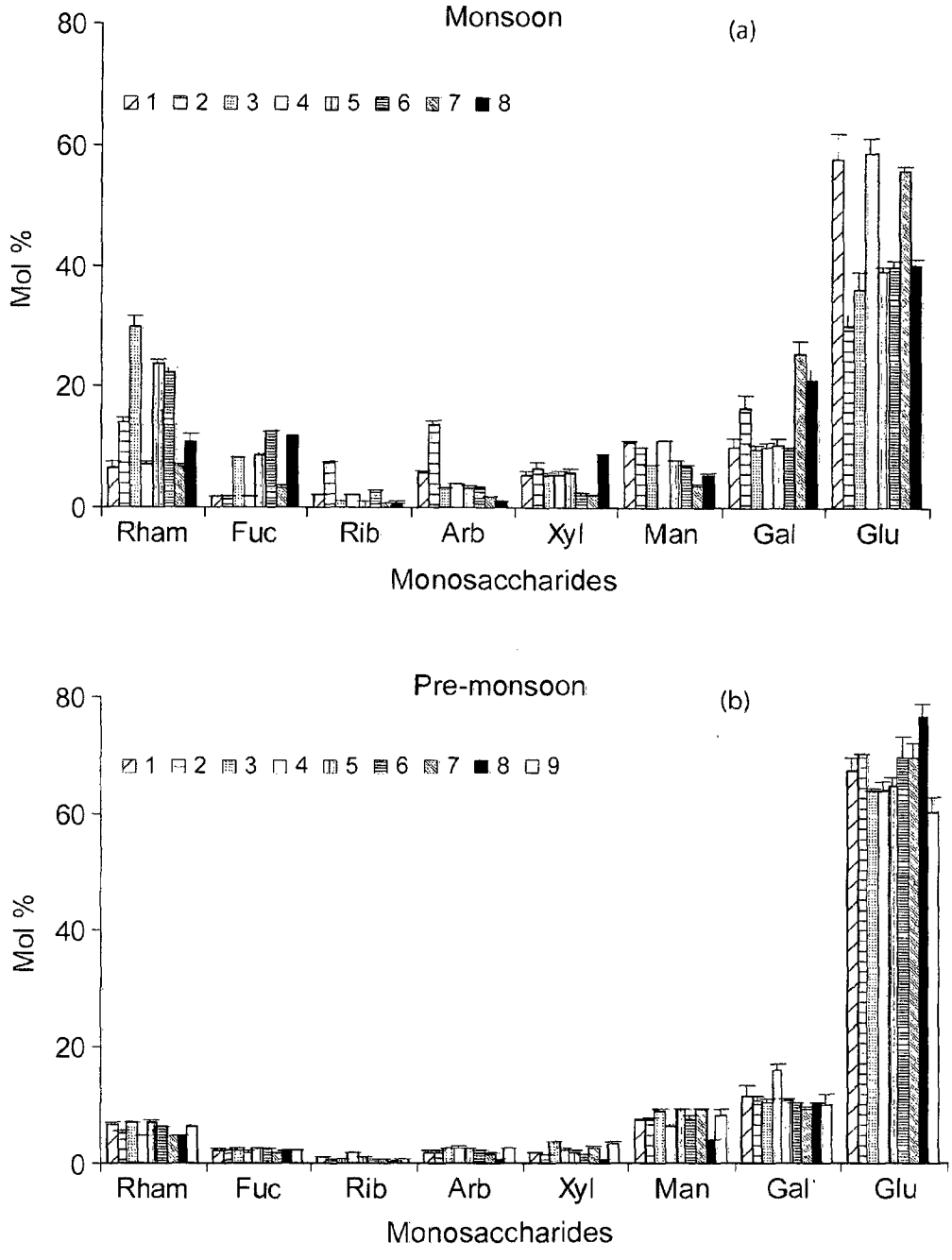


Fig. 4B.4. Variation in the monosaccharide composition (mol%) of the suspended particulate matter (SPM) collected at various stations during the monsoon (a) and the pre-monsoon (b) in the Mandovi estuary. Bar indicates mean, ±SD. Rham = rhamnose; Fuc = fucose; Rib = ribose; Arb = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; and Glu = glucose.

Rhamnose and fucose are more abundant storage polysaccharides of bacteria, fungi and phytoplankton. Rhamnose plus fucose (glucose free) were more abundant at station #3, #5, #6 and station #8 during the monsoon than pre-monsoon season (Fig. 4B.5a).

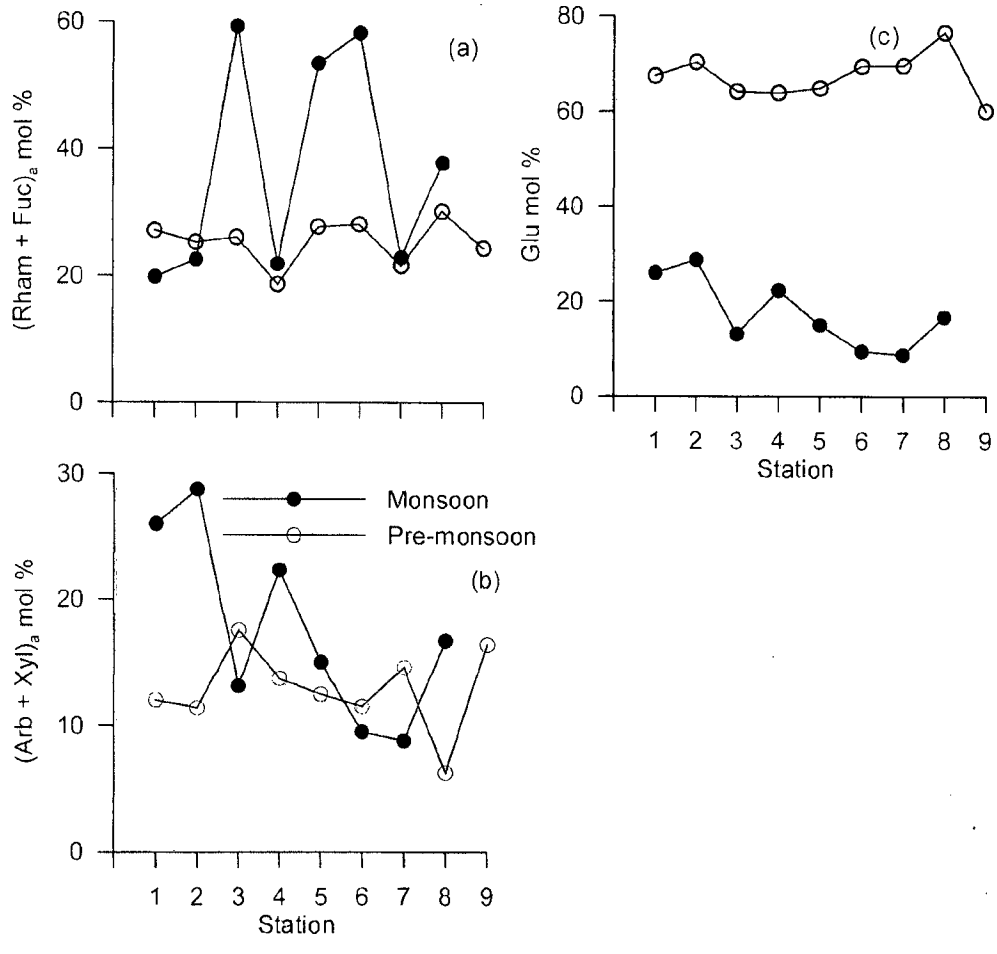


Fig. 4B.5 Seasonal variations in glucose (Glu) free mol % of (Rham + Fuc)a (a), and (Arb + Xyl)a (b) and mol % Glu (c) of the suspended particulate matter (SPM) of the surface water collected from various stations from the Mandovi estuary during the monsoon and the pre-monsoon season.

Glucose was more abundant (60 – 77 mol%) during the pre-monsoon than monsoon (30 – 59 mol%) (Fig. 4B.5c). The higher rhamnose plus fucose was associated with lower glucose during the monsoon season (Fig. 4B.5). The rhamnose plus fucose showed significant ($r = -0.694$, $n = 8$, $p<0.05$) negative relationship with glucose during monsoon. Arabinose and xylose are more abundant in terrestrial plants. The contribution of arabinose plus xylose (glucose free) was more during the monsoon than the pre-monsoon period (Fig. 4B.5b).

4B.3.5 Monosaccharide ratios

Mannose:xylose ratio showed strong seasonal and spatial differences. During the monsoon season, with exception of some values, the ratio appears to decrease from the mouth to the upstream region of the estuary (Fig. 4B.6a). Conversely, during the pre-monsoon season the ratio did not show any trend and was greater than that recorded for the monsoon season.

Hexoses:pentoses ratio also showed temporal and spatial differences (Fig. 4B.6b). With exception of one value, hexoses;pentoses ratio exhibits small variation from the mouth to the upstream region of the estuary during the monsoon season (Fig. 4B.6b). The hexoses;pentoses ratio was higher during the pre-monsoon season.

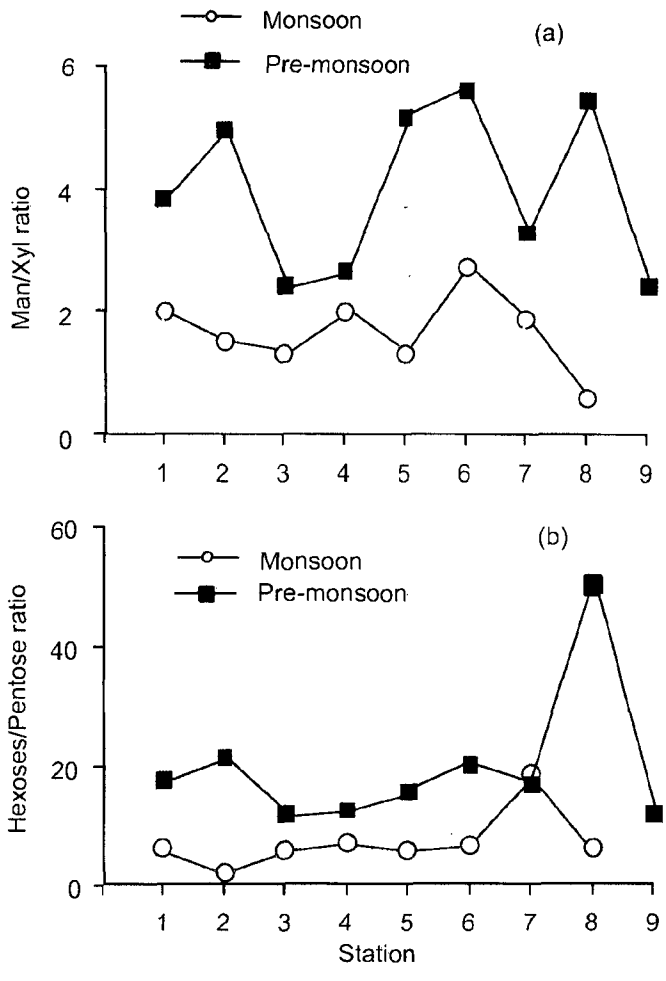


Fig. 4B.6. Variation in the mannose to xylose ratio (a), and hexoses to pentoses ratio (b) of the suspended particulate matter (SPM) of the Mandovi estuary.

Table 4B.3 Factor loading of varimax-rotated factors extracted by PCA, and their eigenvalues and variences

Factor	1	2	3
Rhamnose	-0.7669	0.4038	0.1142
Fucose	-0.8313	-0.0883	0.2857
Ribose	-0.1233	0.7895	-0.0185
Arabinose	-0.0694	0.9186	-0.1517
Xylose	-0.3599	0.7333	0.2235
Mannose	0.0920	0.6502	-0.6639
Galactose	0.0181	-0.0188	0.8987
Glucose	0.5832	-0.6049	-0.3771
Chl *a*	0.8461	-0.4217	0.1222
TBC	0.7063	-0.0440	-0.3959
TPCHO	0.8553	0.0729	0.3195
TPURA	0.7133	-0.5907	0.0130
POC/TPN ratio	0.0485	0.6004	0.4237
POC/Chl *a* ratio	-0.3836	0.7569	-0.2239
Eigenvalues	6.3817	2.6953	1.7992
Variance (%)	45.58	19.25	12.85
Cumulative variance (%)	45.58	64.84	77.69

Chl *a* = chlorophyll a; TBC = total bacterial count; TPCHO = total particulate carbohydrates; TPURA = total particulate uronic acids

4B.4 Discussion

4B.4.1 Variation of SPM, POC, TPN, POC/TPN

During the monsoon season, SPM content is higher at sea-end stations than the river-end stations of the Mandovi estuary. This is in contrast with the general observation that SPM decreased from upstream stations to the stations near the mouth of the estuary. High SPM was also associated with high river discharge and low salinity. High wind speed (5.1 to 5.6 m s^{-1}) and tides (2 to 2.5 m) are generally observed during the monsoon

season. The high SPM content during the monsoon season can be ascribed to re-suspension of sediments due to strong westerly winds, wind induced waves and currents and stirring by tidal currents. In contrast, the pre-monsoon season, the SPM content did not show any consistent trend from river end stations to sea-end stations. River discharge is negligible, and weak easterly winds with a speed of 1.8 to 2.6 m s^{-1} prevail during the period. Absence of re-suspension due to weak winds and currents and low river discharge may be responsible for the low SPM content during the pre-monsoon season.

POC and TPN concentrations recorded for the Mandovi estuary are in the range of values recorded for other estuaries (Ittekkot and Arain 1986; Shevchenko et al., 2005; Cai et al., 2008). When expressed as percentage of SPM, POC and TPN concentrations decreased from river end stations to sea end stations both during the monsoon and pre-monsoon seasons. Similar relationships were observed by Meybeck (1982) and Ittekkot and Arain (1986). As explained above, strong westerly winds, tides and currents re-suspend the bottom sediments at the sea-end stations resulting in dilution of both allochthonous (plants and woody materials) and autochthonous (river plankton) organic matter by minerals and clays from upstream areas due to increased rates of erosion. In addition, a reduction in the autochthonous input may be expected during high sediment discharge period since reduced light penetration during this period will reduce primary production (Thurman, 1985). The % POC/SPM showed some differences for the monsoon season (3.6% to 12.6%) and the pre-monsoon season (5.6% to 14.0%) (Fig. 4B.1c). For the Mandovi

estuary, the contribution of POC to SPM was higher than that recorded for the river Indus (0.5% to 2.5% of SPM) (Ittekkot and Arain, 1986). These values are also higher than those reported for the Yangtze estuary (0.5% to 2.5% of SPM) (Wu et al., 2007). A statistically significant relationship exists between SPM and POC ($r = 0.679$; $n = 17$; $P < 0.01$). POC also showed significant positive relationship with TPN ($r = 0.602$; $n = 17$; $P < 0.01$). These relationships suggest that bulk organic material shares a common mechanism that delivers SPM, POC and TPN to the water system. It is expected that flood within the upper and middle reaches would supply a large amount of particulate matter with low POC from drainage basin.

POC/TPN ratio is often used to assess the source and the nature of organic matter. Marine bacteria and phytoplankton have a ratio of <4 to 6 (Hamilton and Hedges, 1988; Elser et al., 2000). In contrast, the POC/TPN ratios for the terrestrial organic matter fall in the range of 12 to 200 (Hedges et al., 1986). Low POC/TPN ratios indicate the presence of relatively fresh organic matter derived from microalgae and bacteria. Whereas, a high (>10) POC/TPN ratio suggests the presence of degraded organic matter or the presence of terrestrially derived organic matter since POC/TPN ratios of terrestrially-derived organic matter and aerosols are generally very high (> 10). During the monsoon season, with the exception of two values, the POC/TPN ratios are relatively high (8 to 22) indicating the presence of degraded organic matter or the presence of terrestrial carbon. River discharge is relatively high during the monsoon season. Thus large amounts of SPM are brought into the Mandovi estuary during

the monsoon season. These externally supplied terrestrial materials may be responsible for the observed high POC/TPN ratios in the Mandovi estuary. However, if the high POC/TPN ratios of the SPM were due to terrestrial material then we would expect high abundance of carbohydrates in these samples. This was indeed the case as carbohydrate concentrations and yields were higher in monsoon season than the pre-monsoon season. This suggests that the high POC/TPN ratios were due to the presence of terrestrial organic matter. In contrast, the POC/TPN ratio of the SPM was relatively low (4.5 to 6.1) implying that organic matter was mostly fresh and derived from microalgae and bacteria during the pre-monsoon season.

4B.4.2 POC/Chl a, $\delta^{13}C$ and $\delta^{15}N$

Microalgal abundance in a sample can be assessed by the POC:Chl *a* ratio. The POC:Chl *a* ratio of the fresh microalgae is low (< 40). The POC:Chl *a* ratios recorded for the SPM samples collected during monsoon season were higher (>100) compared to those reported for fresh microalgae, thereby suggesting the abundance of detrital organic matter or degraded terrestrial organic matter except at stations #7 and # 8. In contrast, during pre-monsoon season, the POC:Chl *a* ratios were ~ 50 at most of the stations. These low POC:Chl *a* ratios provide enough evidence to suggest that relatively fresh organic material was present in the SPM during the pre-monsoon season.

The distribution of $\delta^{13}C$ showed some seasonal variations (Table 4B.1). During the monsoon season, $\delta^{13}C$ values were more lighter (-32.1

to -25.1) with most of the values being lighter than -26.1. These values are closer to the isotopic signature of continental C-3 vegetation (-28.9 to -26. 0 $^{0}/_{00}$) (Fig. 4B.2). In contrast, $\delta^{13}C$ values for the POC collected during the pre-monsoon season are closer to those reported for marine plankton (-22 to -19) except the value recorded at station #4. This suggests that the organic matter was mostly derived from marine plankton. The stable isotope ratio is also influenced by diagenetic and metabolic fractionation. The isotopic composition of organic matter can be altered during oxidation within the water column and within sediments. The selective loss of a specific fraction of the total organic carbon that has a different composition to the bulk fraction can create shifts in $\delta^{13}C$ during diagenesis (Benner et al., 1987). In general, a mixture of organic matter derived from marine and terrestrial sources is observed in the Mandovi estuary.

The $\delta^{15}N$ values showed large spatial and seasonal variations (Table 4B.1). During the monsoon season $\delta^{15}N$ values are lighter (-2.9 to 2.4‰) as compared to pre-monsoon (5.9 to 9.6‰) season. The $\delta^{15}N$ values of terrestrial plants ranges from -5 to 18‰ (average ~3‰) and marine $\delta^{15}N$ ranges from 3 to 12‰ (average ~6‰) (Maksymowska et al., 2000). Based on these values, organic matter was derived from terrestrial sources during the monsoon and from marine sources during the pre-monsoon.

4B.4.3 Distribution of carbohydrates

Concentrations of TPCHO and TNPCHO were higher during pre-monsoon, and were associated with higher Chl *a* concentrations. This

indicates that live phytoplankton is major source of TPCHO and TNPCHO. Conversely, during the monsoon season, high TPCHO and TNPCHO concentrations at stations #1, #2 and #3 were associated with high SPM and high POC/TPN ratio suggesting that the OM at these stations was influenced by terrestrial organic matter.

Concentrations of TPCHO and TNPCHO observed in the Mandovi estuary compares well with those reported for other estuarine waters (Bhosle et al., 1985; Ittekkot et al., 1982; Sigleo, 1996; D'souza and Bhosle, 2001). The carbohydrate production by micro-organisms is influenced by several factors, including nutrients, temperature, light, trace metals, and composition of phytoplankton (Mykelstad, 1974; Morris, 1981; Hitchcock, 1983; Sakugawa and Handa, 1985; Hama, 1991; Biddanda and Benner, 1997; Hung et al., 2001; Shin et al., 2003). These factors varied during the monsoon and the pre-monsoon seasons and thus may have influenced the distribution of carbohydrates in the Mandovi estuary. The TNPCHO accounted for 42 to 93% of TPCHO (Table 4B.2). This suggests that a large proportion of the TPCHO consisted of TNPCHO in the Mandovi estuary. D'souza et al. (2005) observed good correspondence ($r = 0.946$, $p < 0.001$) between the TPCHO concentrations estimated by the phenol-sulfuric acid method (Dubois et al., 1956) and the TNPCHO measured by the GC method. The observed differences between TPCHO determined using the spectrophotometric method, and those measured with GC method may be due to the presence of non-aldose carbohydrates such as amino sugars, uronic acids and sugar alcohols (Burdige, 2000).

The TNPCHO/TPCHO ratio decreased with increasing bacterial abundance. This implies TNPCHOs were preferentially utilised as carbon source compared to TPCHO. The concentrations of Chl *a* had significant positive correlation with TPCHO and TPURA during the study period suggesting that phytoplankton influenced the distribution of these compounds.

Contribution of TPCHO-C to POC is defined as the yield. Carbohydrate yield is an important tool to identified degradation state of organic matter (Cowie and Hedges, 1992, Kaiser and Benner, 2008). The yield is high for the freshly derived organic matter and low for degraded OM (Cowie and Hedges, 1992; Skoog and Benner, 1997; Hernes et al., 1996; Khodse et al., 2007). For Mandovi estuary, TPCHO and TNPCHO yield ranged from 7.39 to 21.22 % and 4.64 to 13.96 %, respectively (Table 4B.2). Higher yield in monsoon and its association with high POC/TPN (~11) ratio and low Chl *a* indicates presence of terrestrial or soil organic matter. In contrast, high Chl *a*, and its association with low (~5) POC/TPN ratio indicates that SPM was derived from live phytoplankton. Bacterial abundance showed significant inverse correlation with TPCHO yields but not with TPURA yield. This provides evidence that carbohydrate concentrations were controlled through heterotrophic bacterial activity in the surface waters. Moreover, TPURA compounds are more resistant to bacterial degradation as compared to TPCHO and TNPCHO. Similarly, Khodse et al. (2007) reported that, carbohydrate yield decreased with increasing viable cell count of bacteria in the Bay of Bengal.

4B.4 Distribution of particulate uronic acids (TPURA)

In the Mandovi estuary, TPURA showed seasonal variation (Table 4B.2). Average TPURA concentrations were higher during the pre-monsoon season (0.59 ± 0.18 µM C) than those recorded for monsoon season (0.27 ± 0.09 µM C) (Table 4B.2). As previously discussed in chapter 4A, biological activity including phytoplankton species composition and bacterial biomass in pre-monsoon is much stronger than monsoon season. In Mandovi estuary, TPURA/TPCHO ratio ranged from 3 to 9 % (an average 5% ±2) during monsoon and 6 to 10 % (average 9% ±2) in the pre-monsoon. These TPURA/TPCHO values are within the range reported earlier for the Gulf of Mexico (3-9% of TPCHO) (Hung et al., 2003), and the Dabob Bay, USA (9.9% of TPCHO) (Bergamaschi et al., 1999). However, these values are lower than those (5-23% of TPCHO) reported for the northern Indian Ocean (Khodse et al., 2007) and northern East China Sea (3.3 to 18.3 of TPCHO) (Hung et al., 2009). As a result, the biogenic particles may have been more degraded/ decomposed by bacteria, or high production of uronic acids, which would lead to high surface water TPURA/TPCHO ratios during pre-monsoon (Santschi et al., 2003).

Mandovi estuary, TPURA yields varied from 0.24 to 1.42% of POC (Table 4B.2). Average TPURA yield for Gulf of Mexico was 0.64% and 0.70% in 2000 and 2001, respectively (Hung et al., 2003). TPURA yield for northern Indian Ocean varied from 0.2 to 6.3% (Khodse et al., 2007, 2009). TPURA had significant positive correlation ($r = 0.781$, $p<0.01$) with diatom abundance during pre-monsoon. However, such relationship was

not observed in the monsoon season. From this relationship, it was evident that TPURA abundance was controlled by diatoms in the Mandovi estuary during the pre-monsoon season. Uronic acids are surface active compounds. Therefore, particle adsorption may play important role in controlling the concentration of TPURA in surface waters. This observation was supported by the fact that higher content of TPURA was recorded in sinking particles (Bergamaschi et al., 1999). The significant positive correlation between uronic acid concentration and metal concentration suggest that uronic acids are involved in metal cycling (Hung et al., 2001, 2003).

4B.4.5 *Monosaccharides compositions of particulate matter*

Monosaccharide provides useful information on the origin and cycling of the particulate carbohydrates. Earlier investigations suggested that, no single neutral sugar be directly related to its origin since no monosaccharide is produced exclusively by a single organism (Cowie and Hedges, 1984; Guggenberger et al., 1994a,b, de Cunha et al., 2002). Recently, weight (mol) percentages and the ratios of the neutral sugars have been used to differentiate microbial, vascular plant or planktonic sources in marine waters (Jia et al., 2008). The monosaccharide compositions in the particulate matter varied spatially and seasonally in surface waters of the estuary (Fig. 4B.4). This variability in monosaccharide compositions was due to changes in the sources of organic matter, phytoplankton abundance and compositions, growth phase

of microorganisms and nutrient concentration in the surface waters of the estuary.

Glucose contribution of 20-80% of TNPCHO has been reported for SPM from different marine environments, particularly during phytoplankton bloom or for laboratory grown stationary phase diatom cultures (Ittekkot, 1982; Tanoue and Handa, 1987; D'souza and Bhosle, 2001; Khodse et al., 2007). Glucose was the most abundant (30 to 77%) monosaccharide in the particulate matter of the Mandovi estuary. Glucose is derived from both structural storage compounds of phytoplankton (Haug and Myklestad 1976, Skoog and Benner 1997; Hama et al 2004). Glucose concentration in the SPM was significantly different (ANOVA $p < 0.001$) during monsoon and pre-monsoon season. Low glucose recorded in monsoon, which may indicates preferential removal of glucose rich polymer or dilution by glucose poor material. Arabinose and xylose are not synthesised to a great extent by soil microbes, but are quantitatively important constituents of plant materials (Gugenberger et al., 1994). During monsoon season, arabinose plus xylose concentrations were higher than pre-monsoon indicating the SPM was influenced by terrestrial organic matter during monsoon. Galactose, mannose and especially deoxysugars (Fuc and Rham) are considered to be mainly synthesized by microbes as reserved polysaccharides (Biersmith and Benner, 1998). Deoxy sugars increased with degradation of organic matter (Panagiotopoulos and Sampere, 2005a). For the SPM samples higher deoxysugars were associated with lower glucose content during the monsoon. This suggests that deoxysugars are derived from degraded organic matter. During monsoon

season mol% rhamnose plus fucose showed significant negative relationship ($r = -0.694$, $n = 8$, $p<0.05$) with mol% glucose. This indicates that during degradation, the decrease in glucose (mol%) was associated with increase in deoxysugars which are microbial biomarkers.

The mannose to xylose ratio is useful to differentiate inputs from angiosperms and gymnosperm tissues (Cowie and Hedges, 1984). Xylose content is higher in angiosperms than the gymnosperms (Cowie and Hedges, 1984). Xylose allows clear distinction of angiosperms not only from gymnosperm but also from plankton and bacteria. Mannose content is higher in gymnosperms than angiosperms (Cowie and Hedges, 1984). The observed mannose/xylose ratios were < 2 in monsoon, which are similar to those found in angiosperm leaves and grasses (Cowie and Hedges, 1984). In pre-monsoon season, mannose/xylose ratio varied from 2 to 5.5 suggesting contribution from gymnosperm wood and non-woody tissue of gymnosperm tissues (Cowie and Hedges, 1984). The terrestrial input was also supported by high water discharge, low salinity, high POC/Chl *a* ratio, high POC/TPN ratio and low hexose/pentose ratio and depleted $\delta^{13}C$ values during the monsoon season (Ittekkot and Arain, 1986; D'souza et al., 2003).

4B 4.6. PCA

In order to further differentiate the sources of organic matter some of the biological and chemical (monosaccharide) parameters were used as biomarkers and data were processed using PCA. PCA extracted three factors and explains 78% of the total variability (Table 4B.3). First factor

explained 46% of the variability and showed high positive loading for TPCHO, Chl *a*, TPURA, TBC and glucose indicating marine source of organic matter. Second factor was controlled by arabinose, xylose, ribose, mannose, POC/TPN ratio, and POC/Chl *a* ratio. These components suggest the influence of terrestrial organic matter. Ribose is non-structural and a labile compound (RNA or nucleotides) which may degrade rapidly (Hernes et al., 1996). Ribose, arabinose, xylose and POC/TPN ratio cluster together indicating that the ribose originates during the bacterial reworking of terrestrial organic matter. Mannose found in polysaccharides of diatoms (Bhulikar and Kroth, 2008) as well as in bacterial polysaccharides (Decho, 1990). Third factor accounted for 13% of the variance and was influenced by galactose, fucose and rhamnose which formed a close cluster. Rhamnose and fucose are generally found in extracellular or cell wall polysaccharides of diatom (Tanoue and Handa, 1987; Bhulikar and Kroth, 2008) and in bacteria (Kennedy and Sutherland, 1987) and degraded organic matter (Panagiotopoulos et al., 2005). Galactose is more abundant in diatom cell wall (Hecky et al., 1973). During the monsoon season SPM had low Chl *a*, high POC/TPN and POC/Chl *a* ratio, depleted $\delta^{13}C$, $\delta^{15}N$ of the SPM was associated with high water discharge. This implies that organic matter during the monsoon season was derived from terrestrial sources. In contrast, high Chl *a,* low POC/TPN and POC/Chl *a* ratio, heavier $\delta^{13}C$, $\delta^{15}N$ and low water discharge suggest the presence of fresh organic matter derived from phytoplankton during the pre-monsoon.

4B.5 Conclusion

Monsoon period was characterized with high suspended matter, greater carbohydrate yields, high POC/TPN ratio, and POC/Chl *a* ratio, low Chl *a* concentration, low glucose mol%, low hexoses/pentoses and mannose/xylose ratio. From these parameters it was evident the estuary was influenced by degraded and/or terrestrial organic matter during the monsoon season. In contrast, pre-monsoon showed low POC/TPN and POC/Chl *a* ratio, high glucose content, low discharge and high hexoses/pentoses ratios suggesting the abundance of biogenic organic matter.

Chapter 4C

Distribution of particulate amino sugars in the Mandovi estuary, west coast of India

4C.1 Introduction

Amino sugars (AS) are unusual carbohydrates as they contain nitrogen, and serve as a source of organic carbon and nitrogen in marine system. They are important constituents of dead and living organic matter, and play an important role in the dynamics and cycling of both carbon and nitrogen. AS are derived from many sources including microbial cell wall, peptidoglycan, polysaccharides and lipopolysaccharides (Brock et al., 1994). In aquatic environments many marine organisms such as copepods and other crustaceans are rich in chitin, a polymer, of N-acetyl glucosamine (Benner and Kaiser, 2003; Devis et al., 2009). Chitin is one of the abundant polymers in marine environments. Glucosamine (GlcN), galactosamine (GalN), and manosamine (ManN) are important constituents of diatom cells, whereas muramic acid (Mur) is a component of bacterial cell walls (Benner and Kaiser 2003). Therefore, AS are widely distributed in dissolved, suspended and sedimentary organic matter (Muller et al., 1986; Ittekkot et al., 1984; Haake et al., 1993; Dauwe and Middelburg, 1998; Benner and Kaiser, 2003; Fernandes et al., 2006; Niggemann and Schubert, 2006; Kaiser and Benner, 2008; Tremblay and Benner, 2009). AS are routinely used to characterize source of organic matter. For example, high GlcN/GalN ratio (>4) indicating the abundance of chitin containing zooplankton, and low ratio (<3) suggest the abundance of bacteria (Muller et al., 1986; Tremblay and Benner, 2009). Peptidoglycan is the only source of Mur. Low concentration of Mur and variable ratio of GlcN/Mur suggests that peptidoglycan remnants are minor constituents of dissolved, particulate and sedimentary organic matter

(Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Kaiser and Benner, 2008). Mur concentration has been used to estimate the abundance of bacteria in particulate organic matter and sediments (King and White, 1977; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Kaiser and Benner, 2008). Amino sugar yields and composition provides useful information on the degradation state of organic matter (Jennerjahn and Ittekkot, 1999; Gupta and Kawahata, 2000).

There is little knowledge about total particulate amino sugars (TPAS) distribution and cycling in marine and estuarine environments. In view of this, the aims of present study were to assess the variability of TPAS concentration and composition in suspended particulate matter of the Mandovi estuary, 2) to use the individual amino sugars and their ratio to indentify the sources of organic matter and processes controlling its nature, and to estimates bacterial-C contribution of particulate organic matter.

4C. 2 Materials and methods

4C.2.1 Sample collection and analysis

Water samples were collected from 9 stations in the Mandovi estuary during the monsoon (August 2005) and pre-monsoon season as described in chapter 4A. Water sample was filtered through 200 µm filter and then filtered on ashed GF/F filters. The filters were transferred into clean vials and stored at -20 °C until analysis. Data on environmental parameters and other bulk organic matter parameters were taken from earlier chapters as and when required.

4C.2.2 Analysis of TPAS

TPAS were determined following the method described in chapter 3B.

4C.2.3 Statistical analysis

Analysis of variance (ANOVA) was carried out to understand the variations of, amino sugars concentrations during monsoon and pre-monsoon seasons in the Mandovi estuary. The ANOVA and correlation analysis were carried out following methods described in earlier chapter 4A.

4C.3. Results

4C.3.1 TPAS concentration and composition

TPAS concentration varied from 62.2 to 173.1 nmol/l and 38.8 to 267.2 nmol/l during monsoon and pre-monsoon seasons, respectively (Fig. 4C.1a). During the monsoon season, TPAS concentrations decreased from the mouth station (station #1) to upstream region (station #8) of the estuary (Fig. 4C.1). High TPAS concentration was recorded at station #8 during pre-monsoon (Fig. 4C.1a). TPAS-C accounted for 0.95 to 3.29% and 1.52 to 4.47% of POC during the monsoon and pre-monsoon season, respectively (Fig. 4C.1b). Similarly, TPAS-N accounted 0.35 to 1.86 % and 0.26 to 1.64% of TPN during the monsoon and pre-monsoon period, respectively (Fig. 4C.1c). TPAS-C/POC (ANOVA, $p<0.01$) and TPAS-N/TPN (ANOVA, $p< 0.001$) showed significant variation during the monsoon and the pre-monsoon seasons. TPAS-C and TPAS-N yield was higher during the monsoon than the pre-monsoon (Fig. 4C.1b,c).

Generally, TPAS-C and TPAS-N yield increased from mouth (station #1) to station # 7 in the upstream region of the estuary and then decreased at station # 8 during the monsoon (Fig. 4C.1b,c).

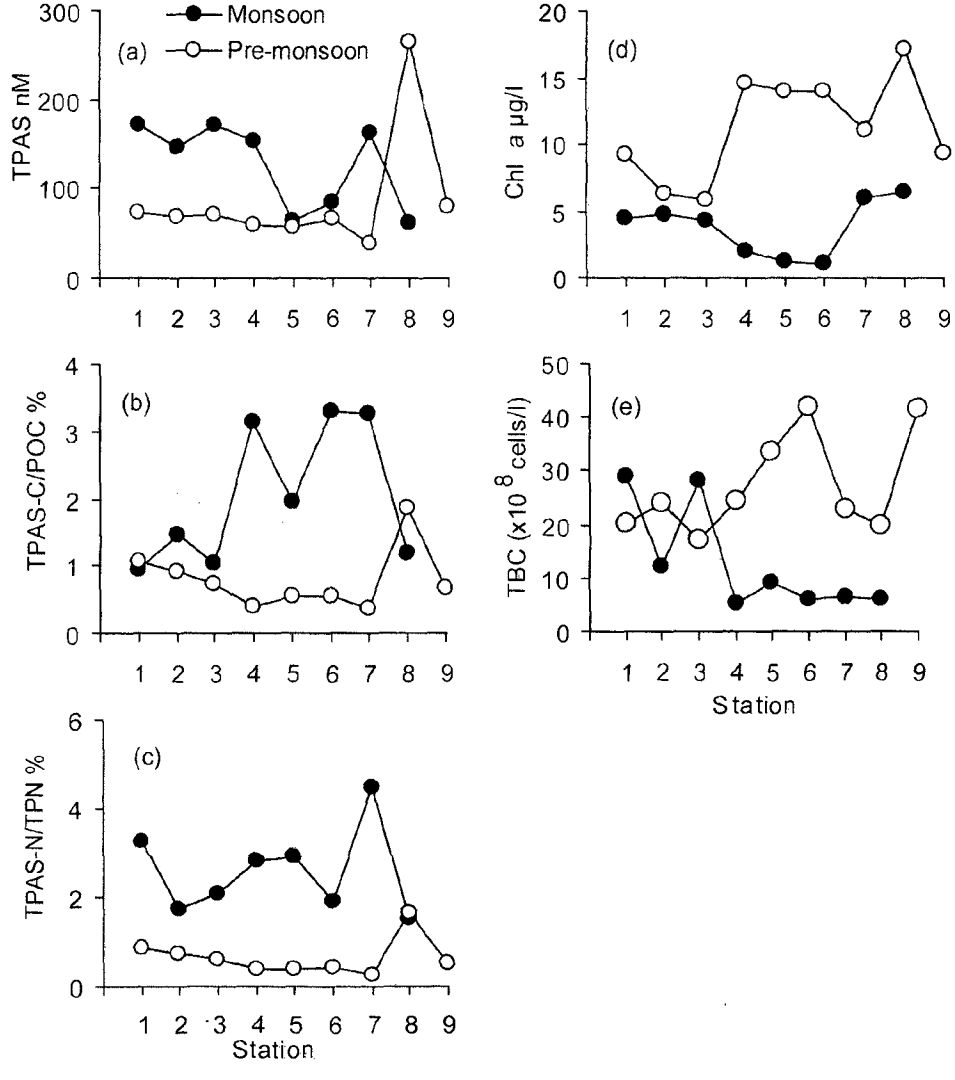


Fig. 4C.1. Variation of total particulate amino sugars (TPAS) (a), total amino sugar carbon yield (TPAS-C) (b), total particulate amino sugar nitrogen yield (TPAS-N) (c), Chl *a* (d) and total bacterial count (TBC) (e) during the monsoon and pre-monsoon seasons in the Mandovi estuary.

In contrast, TPAS-C and TPAS-N yields decreased from mouth (station #1) to station # 7 and then increased at station # 8 during the pre-monsoon season (Fig. 4C.1b, c).

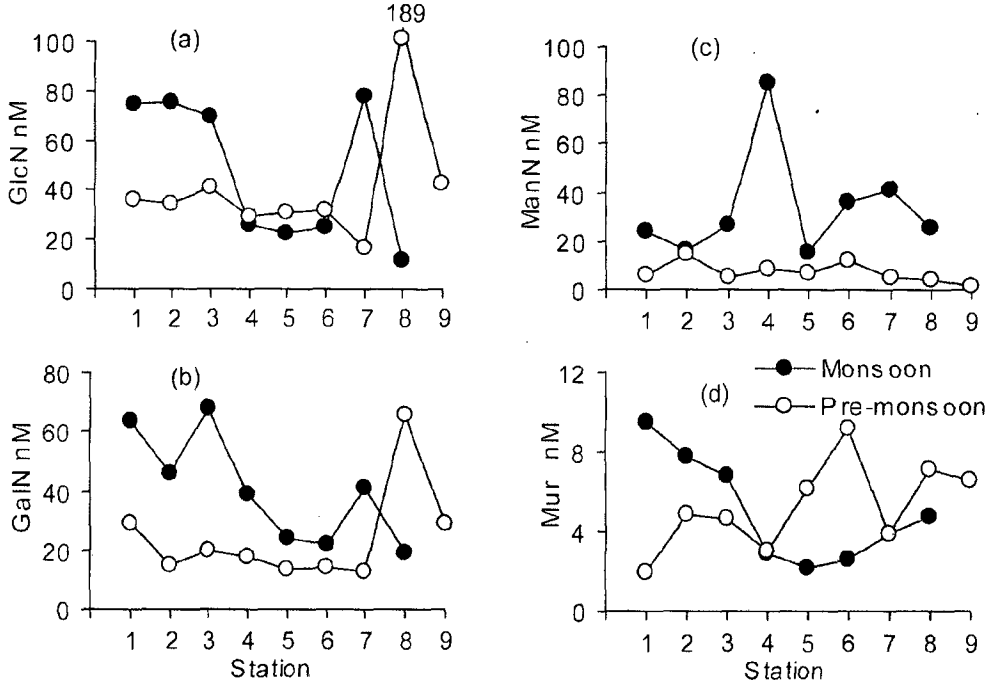


Fig. 4C.2. Spatial variation in concentrations of glucosamine (GlcN) (a), galactosamine (GalN) (b), manosamine (ManN) (c), and muramic acid (Mur) concentration the monsoon and the pre-monsoon seasons in the Mandovi estuary.

Four amino sugars GlcN, GalN, ManN and Mur were detected by using the GC-MS method. Among these GlcN was the most abundant amino sugar in all SPM samples (Fig. 4C.2a). During the monsoon, concentrations of GlcN, GalN, and ManN were higher than those observed in the pre-monsoon (Fig. 4C.2). High GlcN and GalN concentrations recorded at station # 8 during the pre-monsoon (Fig.4C.2). A high

concentration of ManN and Mur was observed at station # 4 and station #1 during monsoon, respectively (Fig.4C.2c,d). Mur concentration was higher in high salinity water during monsoon season (Fig. 4C.2d). In contrast, Mur increased towards the lower salinity water and was associated with high bacterial abundance during the pre-monsoon (Fig. 4C.1e, Fig.4C.2d). GlcN was the most abundant amino sugar as it accounted for 17 to 71 mol% of TPAS.

Table 4C.1. Amino sugar composition (mol%) of suspended particulate matter of the Mandovi estuary during the monsoon and the pre-monsoon seasons.

Station	Amino sugar mol%			
	GlcN	GalN	ManN	Mur
Monsoon				
1	43.6	37.0	13.9	5.6
2	51.9	31.4	11.4	5.3
3	40.8	39.5	15.7	4.0
4	17.0	25.5	55.7	1.9
5	35.1	37.2	24.3	3.3
6	29.8	25.4	41.7	3.1
7	47.5	25.1	25.1	2.4
8	18.6	31.5	42.2	7.7
Pre-monsoon				
1	49.3	39.9	8.2	2.6
2	50.2	21.4	21.2	7.1
3	58.3	28.4	6.8	6.6
4	50.0	30.7	14.2	5.1
5	53.9	23.7	11.8	10.6
6	47.5	21.4	17.5	13.6
7	43.6	33.7	12.5	10.2
8	71.1	24.7	1.5	2.7
9	53.8	36.2	1.8	8.3

GlcN = glucosamine; GalN = galactosamine; ManN = mannosamine; Mur = muramic acid

This was followed by GalN (21.4 to 39.9 mol%), ManN (1.5 to 55.7 mol%). In contrast, Mur was a minor contributor (1.9 to 13.6 mol%) (Table 4C.1). Average mol% of GlcN and Mur were higher during the monsoon whereas, GalN and ManN were higher during pre-monsoon (Table 4C.1). During monsoon ManN concentration (mol%) was 2 fold higher than pre-monsoon (Table 4C.1).

Carbon normalized concentrations of GlcN, GalN, ManN and Mur were higher during the monsoon season as compared to pre-monsoon season (Fig. 4C.3).

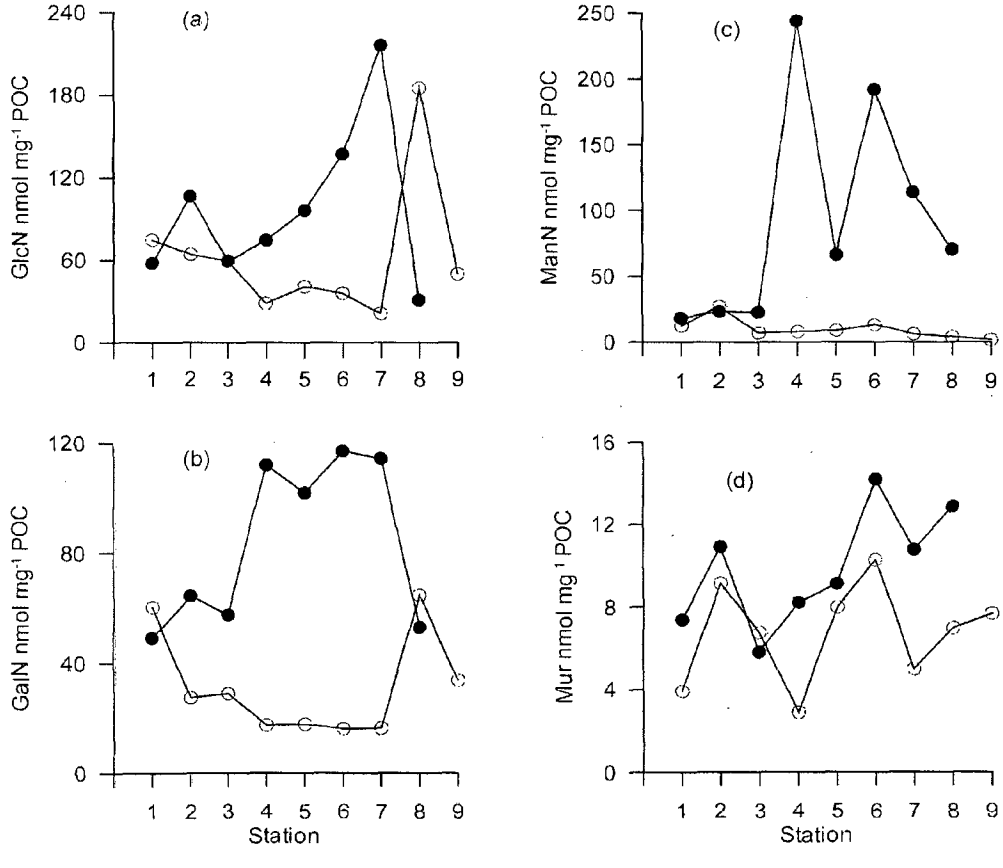


Fig. 4C.3. Variation of carbon normalized concentrations of glucosamine (GlcN) (a), galactosamine (GalN) (b), manosamine (ManN) (c), and muramic acid (Mur) in the suspended particulate matter during monsoon and pre-monsoon seasons in the Mandovi estuary.

Carbon normalized concentrations of GlcN, GalN (except # 8) and Mur generally increased towards the lower salinity waters (Fig. 4C.3). Carbon normalized concentrations of GlcN, GalN and ManN decreased drastically at station # 8 and was associated with low SPM (Fig. 4C.3).

4C.3.2 Amino sugar ratios

GlcN/GalN ratio showed large seasonal variations (0.6 to 2.9) for particulate samples (Fig. 4C.4). The average GlcN/GalN ratio was lower (1.1 ±0.4) for monsoon and higher (1.9 ±0.6) for pre-monsoon. At station #8 GlcN/GalN ratio was higher during monsoon and lower during pre-monsoon (Fig. 4C.4a).

In the Mandovi estuary GlcN/Mur ratios varied from 2.4 to 26.6 and showed large spatial variation (Fig. 4C.4b). GlcN/Mur ratio increased (except # 8) from mouth to fresh water during monsoon while GlcN/Mur ratio decreased during pre-monsoon (Fig. 4C.4b). The GlcN/ManN ratios also showed large variations (0.3 to 47.9), the highest ratio was recorded at # 8, # 9 with high Chl *a* during monsoon (Fig. 4C.4c).

Peptidoglycan and bacterial carbon contribution were higher for the pre-monsoon than the monsoon season (Fig. 4C.4d). Bacterial carbon accounted for 4.7% to 11.9% and 5.9 to 14.6% of POC during the monsoon and pre-monsoon season, respectively (Fig. 4C.4e). Bacterial nitrogen contribution to TN varied from 4.5 to 24.6% and 5.5 to 12.3% during the monsoon and the pre-monsoon, respectively (Fig. 4C.4f).

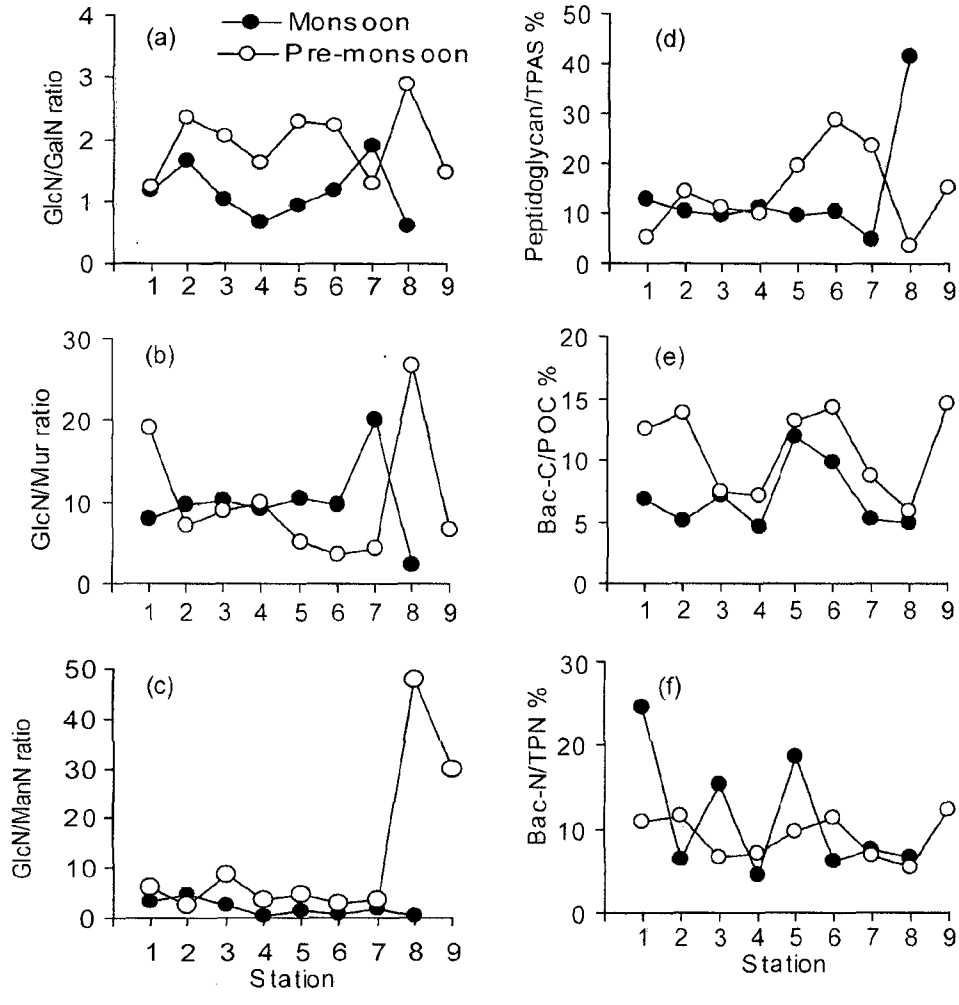


Fig. 4C.4 Spatial and seasonal variation of GlcN/GalN (a), GlcN/Mur (b), GlcN/ManN (c) ratios, and the contribution of peptidoglycan (%) to TPAS (d), bacterial carbon (Bac-C) to POC (e) and bacterial nitrogen (Bac-N) to TPN (f) during the monsoon and pre-monsoon seasons in the Mandovi estuary. Glucosamine (GlcN); Galactosamine (GalN); Manosamine (ManN); Muramic acid (Mur).

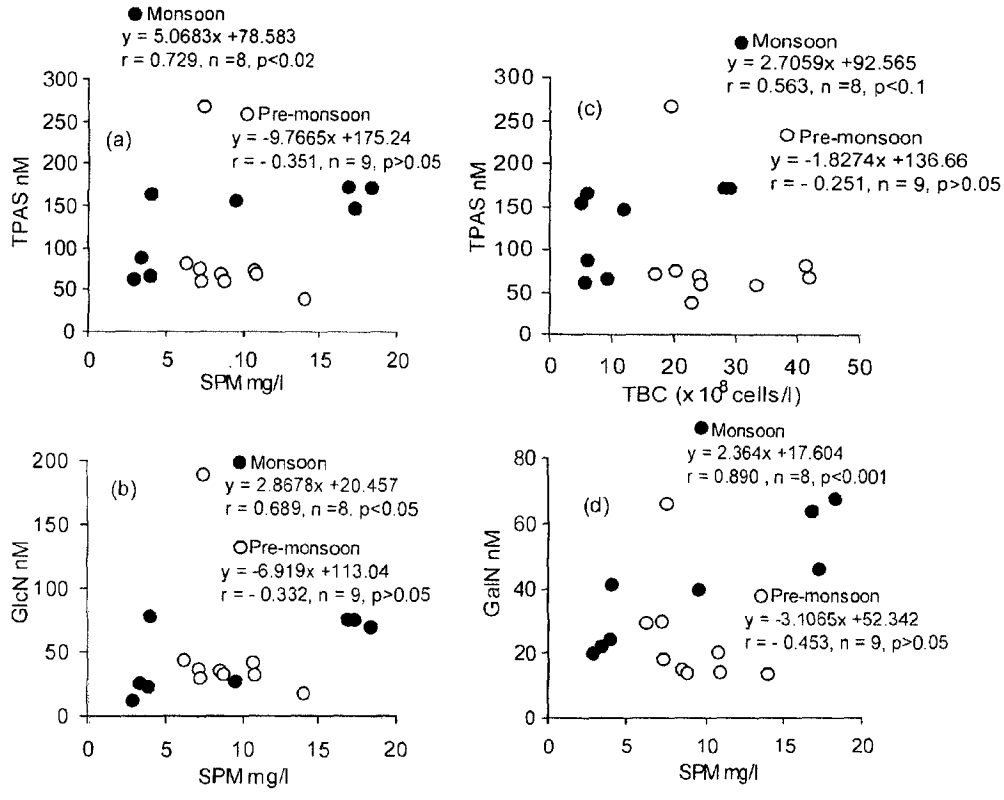


Fig. 4C.5. Relationships of SPM with TPAS (a), particulate GlcN (b) and GalN (d); and the relationship of TBC with TPAS (c) in surface water of the Mandovi estuary.

SPM showed significant positive relationships with TPAS, GlcN and GalN during the monsoon season, however similar relationships were not observed for the pre-monsoon season (Fig. 4C.5). Bacterial abundance and amino sugar showed positive correlation during monsoon and negative during the pre-monsoon season (Fig. 4C.5). Bacteria showed positive correlation with Mur concentration (Fig. 4C.6). TPAS carbon yields had significant inverse correlation with bacterial abundance (Fig. 4C.6).

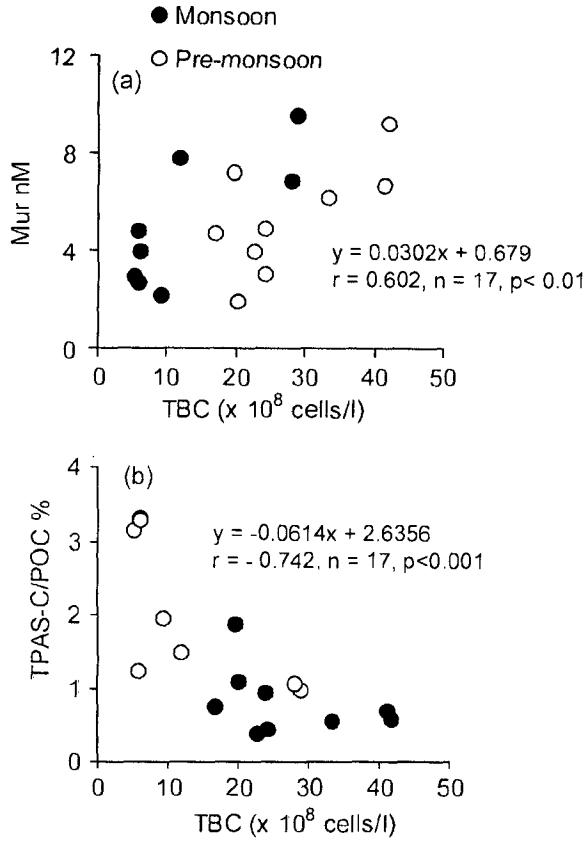


Fig. 4C.6. Relationships of TBC with particulate Mur (a) and TPAS yield (b) in surface water SPM of the Mandovi estuary during the monsoon and the pre-monsoon.

4C.3.3 Quantification of bacterial contribution

Mur yield was used as bacterial biomarker to estimate bacterial contribution to the bulk POC and TPN. This approach assumes that the biomarkers are of similar reactivity as bulk bacterial carbon and nitrogen. The proportion of bacterial carbon (C) or nitrogen (N) was calculated by following equation.

$$\% \text{ Bacterial C or N} = 100\{\text{biomarker}\}_{\text{sample}} / \{\text{biomarker}\}_{\text{bacteria}} \quad \text{-------(1)}$$

Here $(biomarker)_{sample}$ represents the C or N normalized yields of an individual biomarker (Mur) in samples if % bacterial C is estimated and N normalized yields are used for the estimation of % bacterial N. $(Biomarker)_{bacteria}$ represents the C or N normalized yield of the same biomarker in the same assemblages of cultured bacteria (Table 4C.2). In the Mandovi river, SPM sample C and N normalized yield of Mur was lower as compared to the cultured bacteria shown in Table 4C.2. Biomarker (Mur-C and Mur-N) yields calculated from equation 1 were higher (35% ±10% of POC and 62% ±25% of TPN) during the monsoon and lower (24% ±9% of POC and 24% ±8% of TPN) in the pre-monsoon, respectively.

Table 4C.2 Carbon and nitrogen normalized yields of muramic acid in different assemblages of cultured bacteria and Mandovi river particulate organic matter (POM).

Bacterial sources	Mur (nmol mg C^{-1})	Mur (nmol mg N^{-1})
Soil and freshwater [a,b]	42.3 ±6.9	163 ±16.6
Estuary[a,c]	21	107
Seawater[a]	28.1 ±7.1	121.3 ±26.0
Present study	8.2 ±3	51 ±32

Mur = muramic acid; a = Kaiser and Benner (2008); b = Tremblay and Benner (2009); c = Tremblay and Benner (2006).

4C.4. Discussion

4C.4.1 Amino sugars as source indicator

Amino sugars are important component of phytoplankton, bacteria and terrestrial plants and soil organic matter in aquatic environments (Ittekkot and Arain, 1986; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Kaiser and Benner, 2008; Tremblay and Benner, 2009). TPAS concentrations (38.8 to 267.2 nmol/l) in the Mandovi River were lower than those reported (580 nmol/l) for large river such as the Indus (Ittekkot and Arain, 1986). However, TPAS concentrations observed in the Mandovi estuary were higher than those recorded for the oceanic waters (Benner and Kaiser, 2003; Fernandes et al., 2006). TPAS distribution was influenced by SPM and bacterial abundance and not by Chl *a* during the monsoon. This was supported by the positive correlations of TPAS with SPM and TBC (Fig. 4C.5). In contrast, Chl *a* showed positive correlation with TPAS in the pre-monsoon season suggesting the influence of phytoplankton. GalN showed significant positive correlation ($r = 0.871$, $n = 8$, $p<0.001$) with bacterial abundance and SPM ($r = 0.890$, $n = 8$, $p<0.001$) during the monsoon season. This implies that SPM and bacteria were the sources of GalN during the monsoon.

The higher ManN concentration during the monsoon suggests accumulation of the ManN in the SPM. ManN is common in bacteria (Kenne and Lindburg, 1983) and phytoplankton (Benner and Kaiser, 2003). ManN is also widely distributed in membrane glycolipids as a building block of salisilic acids. Carbon normalized concentration of GlcN, GalN, ManN increased towards fresh water region of the estuary

suggesting that fresh water can be a source of TPAS in marine waters (Fig. 4C.3).

4C.4.2 Amino sugar yields

Carbon normalized yields of TPAS are useful to assess the reactivity of organic material. In freshly grown marine microalgae amino sugars accounted for 0.1 to 0.5% and 0.2 to 0.6% of the carbon and nitrogen, respectively (Benner and Kaiser, 2003). Similarly, in phototrophic and heterotrophic bacteria, TPAS accounted for 0.3 to 3.9% and 0.3 to 2.9% of the carbon and nitrogen, respectively (Benner and Kaiser, 2003). Thus the yields of TPAS-C and TPAS-N in marine microalgae and bacteria are low. In contrast, for zooplankton amino sugar yields are relatively higher as was evident from relatively greater contribution of amino sugar-carbon (12%) and amino sugar-nitrogen (7%) to POC and TPN, respectively. Therefore, high concentrations of amino sugars in marine samples mostly indicate the presence of chitin rich organic matter derived from zooplankton. The contribution of TPAS-C to POC and TPAS-N to TPN was higher in the monsoon season during which Chl *a* was low. This suggests that TPAS were derived from detrital and soil material. TPAS-C contribution to POC had significant inverse correlation ($r = -0.742$, $n = 17$, $p < 0.001$) with bacterial numbers implying that amino sugars served as carbon sources for heterotrophic bacteria.

4C.4.3Amino sugar ratios

Amino sugar composition is a useful tool to differentiate the relative inputs of organic matter derived from zooplankton and bacteria in marine waters (Muller et al., 1986; Haake et al., 1993; Benner and Kaiser, 2003; Fernandes et al., 2006; Niggemann and Schubert, 2006). For the Mandovi river particulate samples GlcN/GalN ratio varied from 0.6 to 1.9 for monsoon, and 1.2 to 2.9 for pre-monsoon season (Fig. 4C.4). These ratios are similar to those reported for POM from other marine environments (Benner and Kaiser, 2003; Fernandes et al., 2006). Similarly, GlcN/GalN ratios of the POM of the Mandovi estuary compares well with those reported earlier (0.44 to 6.1) for Ob and Yenisei rivers (Unger et al., 2005).

GlcN is main component of chitin polymer found in zooplankton. Thus GlcN/GalN ratio is < 3 for heterotrophic bacteria, and 4 to 20 for plants, fungi and zooplankton (Benner and Kaiser, 2003, Muller et al., 1986). The observed GlcN/GalN ratios for the Mandovi estuary are < 3 suggesting that the amino sugars in this estuary was mostly derived from bacteria and photosynthetic algae. GlcN/ManN ratios of 2 to 46 and 8 to 18 for bacteria and algae, respectively have been reported (Benner and Kaiser, 2003; Glaser et al., 2004). GlcN/ManN ratio of SPM of the present study showed large variation and ranged from 0.3 to 47.9 (Fig. 4C.4). These ratios are in the range of values reported for bacteria and algae (Benner and Kaiser, 2003). The higher concentration of ManN reflects lower GlcN/ManN ratio in monsoon (Fig. 4C.4). There are two possible explanations for the high ManN abundances; either there are sources with a high ManN content, or there is selective preservation of ManN.

4C.4.4 Bacterial contribution to the organic matter

Carbon normalized concentrations of Mur were highly variable in the Mandovi estuary (Fig. 4C.3). Yields of Mur were also highly variable in marine and freshwater bacteria (Moriarty, 1977; Mimura and Romano, 1985; Glaser et al., 2004; Kaiser and Benner, 2008). There is no evidence that Mur is found in archaea and eukaryotic organisms. However, Mur is exclusively found in peptidoglycan where it occurs in 1:1 ratio with GlcN (Pedersen et al., 2001). Peptidoglycan is a unique cell wall polymer of bacteria. Therefore, concentrations of Mur were used to calculate the contribution of intact peptidoglycan to TPAS. This bacterial cell wall polymer accounted for a small proportion of the TPAS in monsoon (average 13.8 ± 11.4%) and in the pre-monsoon (average 14.6 ± 8.2%) (Fig. 4C.4).

Mur concentration and bacterial abundance followed similar trend in the Mandovi estuary. The Mur concentration showed significant positive correlation ($r = 0.602$, $n = 17$, $p<0.01$) with total bacterial count indicating that bacteria are major source of Mur in SPM samples. GlcN/Mur ratio of SPM varied from 2.4 to 20.1 and 3.5 to 26.6 during monsoon and pre-monsoon, respectively (Fig. 4C.4). The higher GlcN/Mur ratio associated with low bacterial count and high Chl *a* implies that GlcN concentration was affected by phytoplankton abundance.

Bacterial cell numbers obtained from microscopy were converted to bacterial carbon and nitrogen using an average carbon content of 30.2 fg C $cell^{-1}$ and 5.8 fg N $cell^{-1}$ for coastal marine bacteria (Fukuda et al., 1998).

Bacterial carbon accounted for 4.7 to 11.9% and 5.9 to 14.6% of POC during the monsoon and pre-monsoon, respectively (Fig. 4C.4e). Bacterial nitrogen accounted for 6.1 to 24.6% and 5.5 to 12.3% of TPN during the monsoon and the pre-monsoon season, respectively (Fig. 4C.4f). Bacterial carbon and nitrogen contribution of POC and TPN were also calculated using biomarker (Mur) equation (1), and assuming a bacterial biomarker yields of 28.1 nmol mg C^{-1} Mur and 121.3 nmol mg N^{-1} Mur (Kaiser and Benner, 2008). Estimating bacterial contributions to POM in estuaries is challenging because the POM is derived from different sources with different assemblies of bacteria. Based on the biomarker yields (equation 1), bacterial OM represented 24 to 35% of POC and 24 to 62% of TPN. These values are higher than earlier reported (1-4% for bacterial-C and 4-10% for bacterial-N) for the Amazon river (Tremblay and Benner, 2009).

Estimates of bacterial C and N based on Mur were 3 to 5 fold higher than those calculated using bacterial cell count. This variability may be due to two possible reasons. First, most of Mur is associated with non-living bacteria or cell wall fragments. Second reason is that, Mur is also found in cynobacteria (Jones and Yopp, 1979). Therefore, estimates of bacterial C and N based on Mur could be higher than those calculated from bacterial cell count. Moreover, during the sampling period *Trichodesmium* sp. was present in the surface waters (Prabhu Matondkar personal communication) of the estuary. Thus cynobacteria may be another source of Mur in SPM. Presence of cynobacteria may account for higher contribution of bacterial carbon and nitrogen to POC and TN in the Mandovi estuary.

4C.4. Conclusions

Amino sugars concentration in SPM showed spatial and seasonal variation. Seasonal changes in Chl *a* and bacterial abundance influenced GlcN and Mur distribution during the monsoon and pre-monsoon season. Based on amino sugar ratios it is evident that, amino sugars of SPM were derived from bacteria and photosynthetic algae. GlcN/Mur ratio (2.4 to 26.6) suggests that live bacterial cells are minor contributor to SPM.

Chapter 5

Bacterial utilization of high molecular weight size fractions of dissolved organic matter

5.1 Introduction

In the marine environment dissolved organic matter (DOM) stores large amount of biologically reactive organic carbon that is equal to the amount of carbon dioxide (CO_2) in the earth's atmosphere (Hedges, 1992). The sources and cycling of DOM are of considerable importance in the global carbon cycle (Kirchman et al., 2009, Procal et al., 2009). Phytoplankton primary production is the main source of DOM in aquatic environments, whereas allochthonous inputs are important in freshwater systems (Gueguen et al., 2006). DOM concentrations are also influenced by direct release by organisms (Biddanda and Benner, 1997; Hama and Yanagi, 2001), microbial degradation of organic matter (Hellebust, 1965), viral or bacterial lysis of phytoplankton cells (Jumars et al., 1989). Heterotrophic bacteria are major consumers and remineralizers of DOM. In some ecosystems upto 40 to 60 % of autochthonous primary production is cycled through bacteria (Hoch and Kirchman, 1993). The availability of DOM to heterotrophic bacteria depend on factors such as biochemical composition, molecular size, inorganic nutrient concentrations, photochemical transformation and temperature (Amon and Benner, 1996; Rosenstock et al., 2005; Abboudi et al., 2008)

DOM has been fractionated into high molecular weight (HMW) (>1 kDa), and low molecular (LMW) DOM (< 1kDa) size fractions. In the open ocean environments, HMW DOM can contain large, labile polysaccharides derived from microalgae. These polysaccharides are rapidly taken up by bacteria (Amon and Benner, 1994, 1996). However, in coastal, estuarine and freshwater environments influenced by vascular plants, HMW DOM

may also contain refractory compounds such as humic acids which are not readily decomposed (Meyer et al., 1987; Hopkinson et al., 1998). Similarly, the LMW DOM may contain highly labile compounds (Kiel and Kirchman, 1991) as well as refractory compounds that have been extensively processed microbially (Amon and Benner, 1996). It appears that LMW DOM of the river systems supported more bacterial growth (Volk et al., 1997; Meyers et al., 1987), whereas HMW DOM of the open ocean is most bioavailable (Amon and Benner 1994, 1996). Freeman et al. (1990) have reported that HMW DOM (> 1 kDA) inhibits enzyme activity. However, according to Rochelle-Newall et al. (2004) bioavailability of LMW and HMW fractions varied seasonally, and was not constant.

Dissolved carbohydrate (TDCHO) is one of the largest pools of labile DOM in aquatic environments. TDCHO accounts for a significant fraction of DOC pool in both oceanic (20-30%) (Pakulski and Benner, 1994) and estuarine waters (9-60%) (Hung et al., 2001; Khodse et al., 2010). Heterotrophic bacteria use TDCHO as a source of carbon and energy (Hanisch et al., 1996; Amon et al., 2001). The ratio TDCHO-C to DOC is a useful tool to assess the freshness or lability of DOC with relatively greater ratio indicating labile organic matter and vice versa (Amon et al., 2001). Similarly, utilization of uronic acid (DURA) by heterotrophic bacteria has been reported in waters of the Bay of Bengal and the Gulf of Mexico (Hung et al. 2001, Khodse et al. 2007).

In the coastal environment such as Dona Paula Bay, DOM is derived from autochthonous production, sediment pore waters and allochthonous sources. Dona Paula Bay receives allochthonous inputs

from the Mandovi and Zuari estuaries (Wafer et al., 1997). Riverine DOM contain HMW lignin components that are old in age and are resistant to microbial degradation (Guo and Santschi 2000; Wetzel, 2003). Marine DOM is isotopically heavier ($\partial^{13}C_{oc}$~ -22 ‰) and rich in alkoxy carbon with linear molecular structure (Gagosian and Steumer, 1977). In contrast, estuarine DOM is lighter ($\partial^{13}C_{oc}$~ -27 ‰) and rich in aliphatic, aromatic carboxylic carbon (Engelhaupt and Bianchi, 2001).

Studies on bacterial utilization of DOM have mostly focused on HMW (>1 kDa) and LMW (<1 kDa) DOM size fractions. HMW DOM can contain various sizes of organic matter that presumably may differ in nature, source and TDCHO content. Differences in chemical composition of HMW DOM fractions may influence their utilization by bacteria. Our objective was to compare the utilization of two size fractions i.e. LMW (>10kDa to 30 kDa), and HMW (>30 kDa to 0.2 µm) of HMW DOM by natural bacterial population. The question we asked was whether there were differences in the chemical and biochemical composition of various size fractions of HMW DOM, and weather these differences influenced their utilization by natural bacterial populations.

5.2 Materials and methods

5.2.1 Water sample collection and size fractionation of DOM.

Surface (~1 m) water (25 L) was collected using a Niskin sampler (5L) during the monsoon season (September, 2007) from the Dona Paula Bay (15^0 27' N, 73^0 48' E), west coast of India. Filtration of water sample and size fractionation of DOM were carried out following the procedure

described earlier (Fernandes et al., 2007, Khodse et al., 2008). Water sample was filtered through 200 µm nylon screen to remove large zooplankton, and then filtered through pre-ashed GF/F filter (47 mm, 0.7 µm, Whatman) followed by polycarbonate filter (47 mm, 0.22 µm, Nucleopore). Various steps involved in size fraction of the DOM are shown in Fig. 5.1. The sample was filtered through an 30 kDa MW cut-off cellulose membrane ultra-filter (#YM-30) using a Amicon Ultra-filtration system equipped with a stirred cell until 40 ml remained. Concentrated sample retained on the 30 kDa ultra-filter was defined as high molecular weight (HMW, >30 kDa to 0.22 µm) fraction of DOM. Filtrate from the 30 kDa ultra-filter was then filtered through 10 kDa MW cut-off ultra-filter (#YM-10) until 40 ml remained. Concentrated sample retained on 10 kDa ultra-filter was defined as low molecular weight (LMW, >10 kDa to 30 kDa) fraction of DOM. Both fractions were desalted using UV-Milli-Q water that was passed through a 10 kDa ultra-filter. In general the working pressure in the stirred cell of the ultra filtration unit was maintained at ~30 Psi using AR grade nitrogen gas. Subsequently, the size fractionated water samples were stored at – 20 °C until analysis. Before use, the ultra-filters were cleaned with UV-Milli-Q water, 0.1 N NaOH and then with UV-Milli-Q water, and rinsed 3 times with 100 ml of sample water. Teflon tubing was cleaned with 1 N HCl, and rinsed several times with UV-Milli-Q water. Before use all the glassware was cleaned with dirchromic acid, and rinsed several times with UV-Milli-Q water.

5.2.2 Growth medium

A basal salt solution (BSS) containing (g L^{-1}) 25.0 NaCl, 0.75 KCl, 7.0 $MgSO_4$, 0.019 NH_4Cl, 0.2 $CaCl_2$, 0.70 K_2HPO4,0.3 KH_2PO_4, and 1 ml trace metal solution, pH 7.5 (Jain and Bhosle, 2009) was used as a growth medium to assess degradation of different size fractions of DOM. Three flasks, each containing 400 ml of the BSS medium were sterilized at 120 °C and 15 lb pressure for 10 min. To one of the flasks, 40 ml of the HMW DOM (340 µM-C final concentration), to another 40 ml LMW DOM (261 µM-C final concentration), and to the third flask 40 ml of 0.22 µm filter sterilized BSS (34 µM-C) was added and was treated as a control.

5.2.3 Preparation of the inoculum

In order to prepare natural bacterial inoculum, 500 ml surface water (~1 m) from the Dona Paula Bay was first filtered through GF/F glass filter. (0.7 µm). Filtered water (500 ml) containing natural bacterial population was concentrated to 100 ml by filtering through polycarbonate (0.22 µm) filter (Amon and Benner, 1996; Young et al., 2004, 2005). Concentrated water containing natural bacterial population was used as source of inoculum.

5.2.4 Utilization of the HMW and LMW DOM size fractions

A control flask and flasks with HMW and LMW DOM prepared as above were inoculated with 5 % of the natural bacterial inoculum prepared as above. Sub-samples from each of the flasks were transferred to 60 ml oxygen bottles and incubated without shaking to measure respiration. Flasks and the oxygen bottles were incubated in dark at room temperature (28±2 °C) over a period of 15 d. All the flasks were shaken (100 RPM) using an orbital shaker.

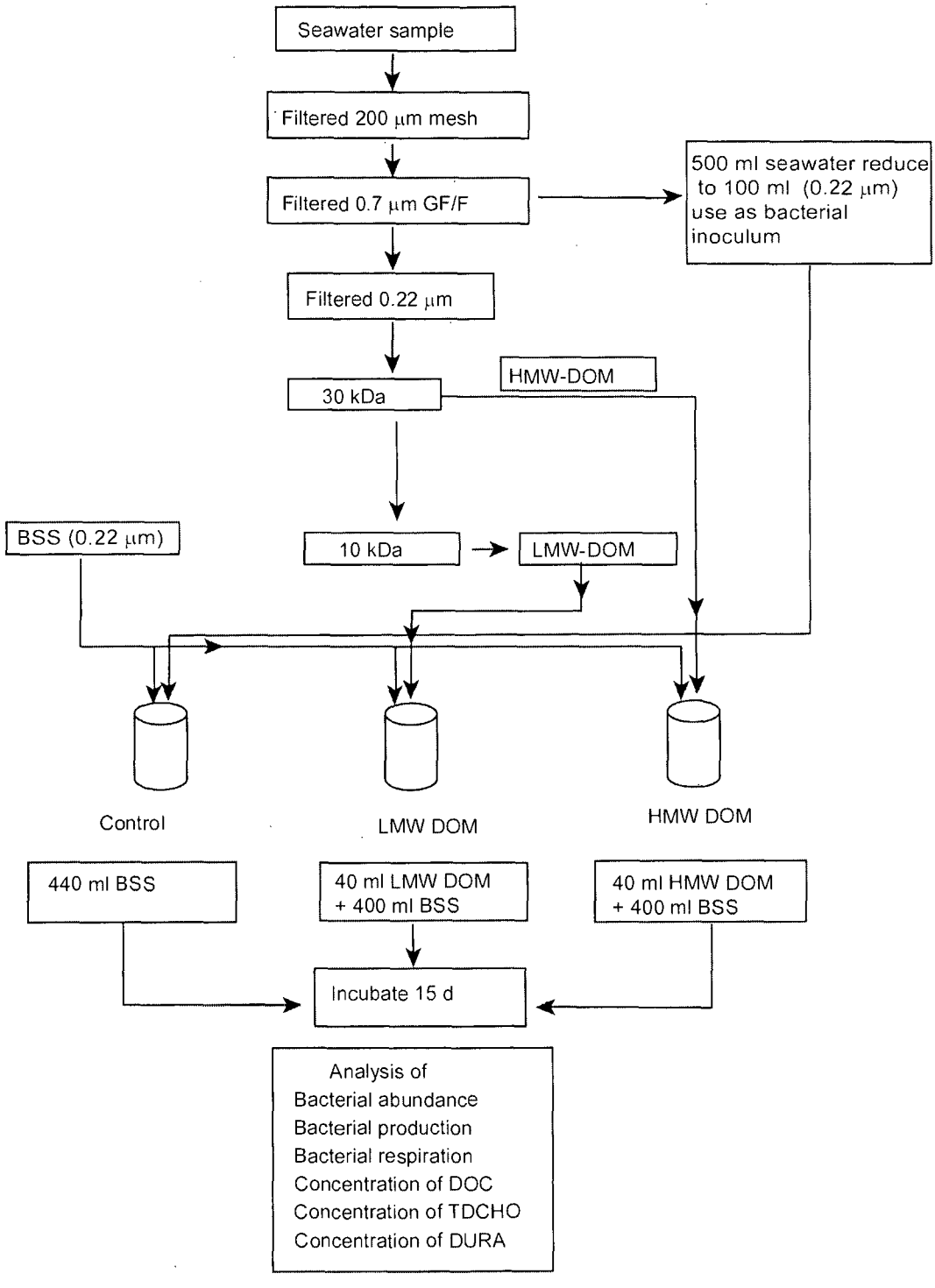


Fig. 5.1 Protocol for DOM size fractionation and its degradation by bacteria.

Oxygen samples were removed at 0, 4, and 8 d following inoculation. Sub-samples for bacterial and chemical analyses were removed from each flask at 0, 2, 4, 8, 10 and 15 d following inoculation. The samples were

then analysed for bacterial cell count (TBC), bacterial productivity (BP), bacterial respiration (BR), dissolved organic carbon (DOC), $\delta^{13}C$, total dissolved carbohydrate (TDCHO), monosaccharide (MCHO) and dissolved uronic acids (DURA).

5.2.5 Sample analyses

DOC was determined following the method described in chapter 4A. For $\partial^{13}C_{oc}$ composition, DOM samples were added in tin capsules and dried at 60 °C in a oven. The dried DOM samples were analysed for $\delta^{13}C$ following the method described in chapter 4B. MCHO, dissolved polysaccharides (DPCHO), TDCHO, and DURA were determined following the methods described in chapter 2. Bacterial abundance (TBC) was estimated following the DAPI (4,6 diamidino- 2 phenylindole) staining method (Porter and Feig, 1980). A known volume of seawater (2 to 5 ml) was stained with DAPI (final concentration 0.01%) for 5 min, and filtered onto 0.22 µm black Nuclepore polycarbonate filter. Bacterial cells were counted in at least 25 randomly selected fields using 100X objective, and an epifluorescence microscope (Nikon). Average cell number per field was calculated, and used to estimate total bacterial cells following the procedure described by Porter and Feig (1980). Bacterial biomass (BB) was calculated from bacterial cell numbers and assuming a bacterial carbon content of 15 fg C $cell^{-1}$ (Coron et al., 1995). Bacterial production (BP) was estimated from the incorporation rate of nucleoside $^{3}H_1$ thymidine into bacterial DNA as described in earlier chapter 4A.

Bacterial respiration (BR) rates were determined by following the decrease in dissolved oxygen (DO). Concentration of DO was measured following the Winkler method described by Parsons et al. (1984). Respiration rates were calculated using the following equation

$$r = Oi - O_f/t \quad (1)$$

r is the respiration rate, O_i , is the initial oxygen concentration, O_f is the oxygen concentration at day 4 or at day 8 following inoculation, and t, is the incubation time (h). Bacterial growth efficiencies (BGE) in the different size fractions of DOM were calculated from bacterial C production and bacterial respiration using the equation given below

$$BGE = (BP/DOC) X100 \quad (2)$$

OR

$$(BP/TDCHO+DURA) X 100 \quad (3)$$

5.3 Results

5.3.1 Organic matter processing

Bacterial abundance and the Initial concentrations of DOC, TDCHO, DPCHO, MCHO and DURA in the source seawater and the various size fractions are shown in Table 5.1. HMW DOM (>30 kDa to 0.2 µM), LMW-DOM (>10Kda to 30 kDa), and very low molecular weight (VLMW, <10 KDa) DOM accounted for 7.8%, 6.1 and 63.7 %, respectively of the source water DOC. Mass balance calculations indicated that 78% of the DOC, 79 % of TDCHO and 85% of DURA could be recovered from the source water using the ultra filtration technique (Table 5.1).

Table 5.1. Dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), dissolved uronic acids (DURA), dissolved monosaccharides (MCHO) and dissolved polysaccharides (DPCHO) and bacterial cell count (TBC) in the surface seawater of the Dona Paula Bay; and recovery of DOC, TDCHO and DURA after ultrafiltration.

Sea water composition	Before size fraction µM C	After size fractionation µM C Fractions			Total (sum) after size fractionation µM C	Recovery %
		>30 kDa to 0.2 µm	>10 kDa to 30 kDa	<10 kDa		
DOC	270.8 ±11.8	21.1 ±0.6	16.4 ±0.6	172.5 ±10.6	210.0	78
TDCHO	44.7 ±2.9	4.1 ±0.6	6.2 ±0.2	25.1 ±1.7	35.4	79
DURA	18.5 ±3.4	2.5 ±0.1	2.4 ±0.2	10.7 ±0.4	15.6	85
MCHO	7.2 ±1.1	-	-	6.6±0.8	-	-
DPCHO[a]	37.5 ±1.8	-	-	18.5±0.9	-	-
TBC ($\times 10^7$ cells/ml)	6.9	-	-	-	-	-

a = TDCHO-MCHO; - = no data.

The observed recovery of the TDCHO compares well with those (68-87%, average= 80%) reported earlier (Khodse et al., 2008). This means contamination or losses were minor during the ultra-filtration process.

5.3.1 Chemical characteristics of DOM size fractions

There were apparent differences in the chemical composition of HMW and LMW fractions of the DOM (Table 5.2). At time 0, LMW DOM fraction contained 261 µMC, 65.6 µMC and 19.4 µMC of DOC, TDCHO, and DURA, respectively (Table 5.2). Similarly, at time 0, HMW DOM fraction contained 340 µMC, 51.7 µMC and 24.0 µMC DOC, TDCHO and DURA, respectively. Thus, HMW DOM fraction had higher concentrations of DOC and DURA and lower concentrations of TDCHO than the LMW fraction (Table 5.2). The relative contribution of TDCHO-C to DOC was higher for LMW DOM fraction as compared to the HMW DOM (Fig. 5.2a). DOC accounted by DURA-C of HMW and LMW fractions was similar (Fig. 5.2 b). $\delta^{13}C$ values varied from – 27.0 ‰ to -26.2 ‰ and from -23.7 ‰ to -21.8 ‰ for HMW and LMW DOM size fractions, respectively (Table 5.2).

5.3.2 Utilization of DOC and variation in $\delta^{13}C$ values

DOC concentrations decreased from 340 to 296 µM C, and from 261 to 171 µM C in HMW and LMW DOM fractions, respectively over the period of incubation (Table 5.2). Bacterial utilization of HMW DOC was low (~13%) as compared to LMW DOC (~35%) (Table 5.3). DOC utilization rates were higher for the LMW DOM than HMW DOM (Table 5.4). $\delta^{13}C$ values of HMW DOM and LMW DOM showed significant differences.

Table 5.2. Changes in dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), free monosaccharides (MCHO), dissolved polysaccharides (DPCHO), dissolved uronic acids (DURA) and carbon isotopes ($\delta^{13}C$) of HMW and LMW fractions over the incubation period.

Incubation	DOC µM C			TDCHO µM C			MCHO µM C			DPCHO µM C			DURA µM C			$\delta^{13}C$ (‰)		
period (d)	Control	HMW	LMW	Control	HMW	LMW	Control	HMW	LMW	Control	HMW	LMW	Control	HMW	LMW	Control	HMW	LMW
0	34±3	340±21	261±28	1.2±0.3	51.7 ±2.7	65.6 ±2.5	0.0	1.1±0.3	1.5±0.9	1.2±0.1	50.6±2.5	64.1±1.4	-	24.0 ±5.4	19.4 ±1.7	-	-26.9	-21.7
2	36±2	328±10	221±33	2.6±0.4	51.5 ±2.5	38.6 ±8.8	0.0	1.7±0.2	5.3±0.7	2.6±0.3	49.8±2.3	33.2±9.5	-	22.1 ±5.3	19.3 ±3.2	-	-26.5	-23.5
4	39±4	313±7	208±12	3.4±0.4	42.7 ±2.1	24.3 ±7.8	0.5±0.1	3.4±0.3	5.9±0.2	2.9± 0.4	37.7±2.4	18.4±3.6	-	27.1 ±3.6	17.7 ±1.8	-	-26.3	-23.1
8	40±3	302±13	184±12	2.7±0.5	41. 0 ±3.7	18.6 ±1.4	0.7±0.1	3.3±0.4	7.1±1.0	2.0±0.2	39.4±3.2	11.5±2.6	-	22.7±0.9	16.5 ±3.5	-	-26.3	-23.7
10	38±6	298±23	181±27	2.6±0.2	40.1 ±0.7	15.8 ±2.5	0.5±0.2	3.9±0.4	6.8±0.8	2.1±0.1	36.2±1.3	9.0±0.1	-	21.8 ±3.0	14.0 ±2.8	-	-26.2	-21.7
15	43±5	296±10	171±18	1.4±0.4	27.7 ±3.7	16.7 ±2.3	0.3±0.1	3.7±0.3	4.3±0.2	1.1±0.2	24.0±41	12.4±2.4	-	19.5 ±4.9	12.4 ±0.9	-	-27.0	-21.8

- = not detected

$\delta^{13}C$ values of HMW DOM showed small differences. However, for LMW DOM, $\delta^{13}C$ values decreased from -21.7 at d 0 to - 23.7at d 8, and then increased again for the remaining period of incubation (Table 5.2).

5.3.3 Utilization of HMW and LMW TDCHO fractions

Over the period of incubation, TDCHO concentrations decreased in both size fractions of the DOM (Table 5.2). HMW-TDCHO decreased from 51.7 µM C at d 0 to 27.7 µM C at d 15, whereas the LMW-TDCHO decreased from 65.6 µM C at day 0 to 15.8 µM C at d 15 following inoculation (Table 5.2). About 75 % of LMW and ~ 46 % HMW TDCHOs were degraded over the period of incubation (Table 5.3).

Table 5.3. Utilization (as % of initial concentration) of dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), dissolved uronic acids (DURA) of HMW and LMW DOM fractions over the period of incubation.

Incubation	DOC		TDCHO		DURA	
period (d)	HMW	LMW	HMW	LMW	HMW	LMW
0	0.0	0.0	0.0	0.0	0.0	0.0
2	3.5	15.3	0.4	41.2	7.9	0.5
4	7.9	20.3	17.4	63.0	-12.9*	8.8
8	11.2	29.5	20.7	71.6	5.4	14.9
10	12.4	30.7	22.4	75.9	9.2	27.8
15	12.9	34.5	46.4	74.5	18.8	36.1

* = DURA production

Bacterial utilization rates of TDCHO were significantly higher (ANOVA, $p<$ 0.01) for the LMW DOM than the TDCHO of the HMW fraction (Table 5.4).

DPCHO concentrations in both fractions showed significant differences (ANOVA, $p<0.01$). DPCHOs of both LMW and HMW DOM were degraded by bacteria, and MCHOs were produced (Table 5.2). The decrease in concentrations of TDCHO or DPCHO was associated with an increase in MCHO concentrations over the period of incubation. The production of MCHO was highest at d 8 and at d 10 for the LMW and HMW fractions, respectively following inoculation (Table 5.2). MCHO/TDCHO ratio increased with the incubation period and was higher in LMW fraction than HMW fraction (Fig. 5.2c).

Table 5.4. Utilization rates (μg C l^{-1} h^{-1}) of dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO) and dissolved uronic acids (DURA) of HMW and LMW DOM fractions over the period of incubation.

Incubation	DOC		TDCHO		DURA	
period (d)	HMW	LMW	HMW	LMW	HMW	LMW
0	0.00	0.00	0.00	0.00	0.00	0.00
2	3.00	10.00	0.05	6.75	0.42	0.02
4	3.38	6.63	1.13	5.16	-0.34*	0.19
8	2.38	4.81	0.67	2.94	0.07	0.16
10	2.10	4.00	0.58	2.49	0.10	0.24
15	1.47	3.00	0.80	1.63	0.13	0.21

*= DURA production

5.3.4 Utilization of HMW and LMW DURA fractions

DURA concentrations in LMW and HMW size fractions of DOM were not significantly different (ANOVA, $p > 0.05$) (Table 5.2). Over the period of incubation, DURA concentrations decreased from 24.0 to 19.5 μM C (19

% degradation) in HMW and from 19.4 to 12.4 μM C (36 % degradation) in LMW DOM. Bacterial utilization rates for DURA were higher for LMW DOM than HMW DOM with the exception of one value (Table 5.4).

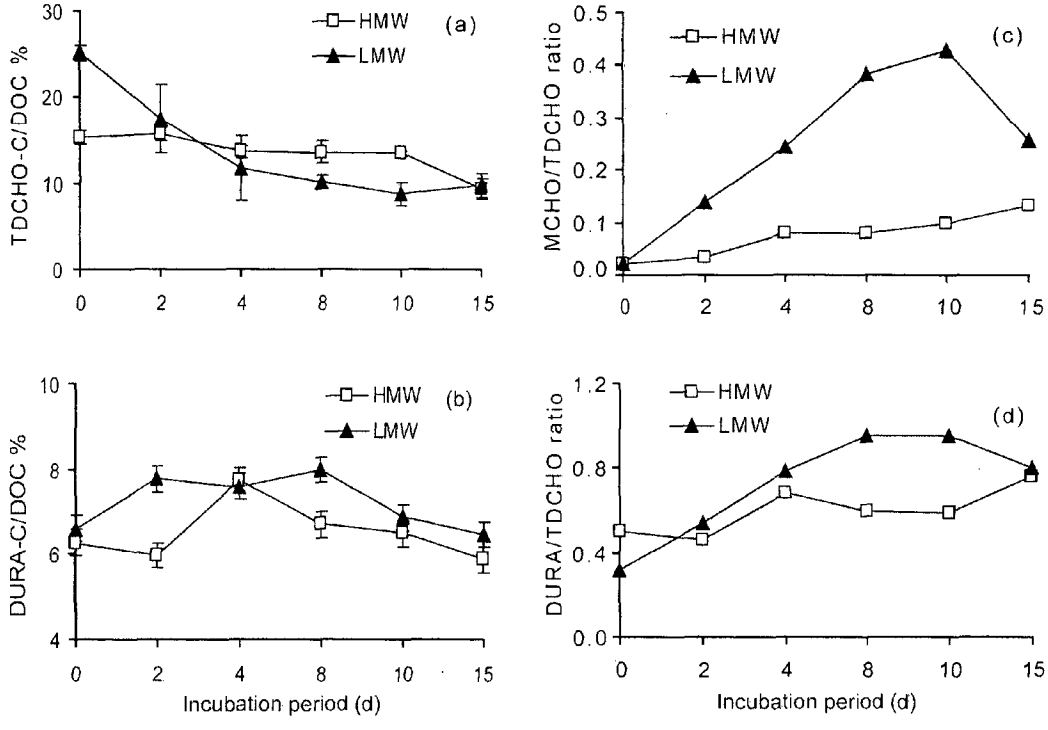


Fig. 5.2. Changes in % TDCHO-C/DOC (a), % DURA-C/DOC (b), MCHO/TDCHO ratio (c), and DURA/TDCHO ratio (d) during the bacterial degradation of HMW and LMW DOM. TDCHO: total dissolved carbohydrate; DURA: dissolved uronic acid; MCHO: free monosaccharide; DOC: dissolved organic carbon.

DURA/TDCHO ratio increased with degradation of DOM and the ratio was relatively higher in LMW fraction than HMW fraction implying preferential removal of TDCHO of LMW DOM (Fig. 5.2d). TBC showed significant positive correlations with DURA/TDCHO ratio of LMW ($r = 0.949$, $p<0.001$) and HMW ($r = 0.798$, $p<0.02$) DOM fractions.

5.3.5 Changes in TBC, BP and BR

TBC, BP and BR were strongly affected by the nature and the source of the DOM size fractions (Fig. 5.3). Both TBC and BP were higher in LMW-DOM than in HMW-DOM (Fig 5.3a, b). When grown on HMW-DOM, TBC gradually increased from 1.6×10^{10} cells L^{-1} at d 0 to 2.2×10^{10} cells L^{-1} at d 4 and then remained nearly same over the remaining period of cultivation (Fig. 5.3a). For LMW-DOM, TBC increased from 1.7×10^{10} cells L^{-1} at d 0 to 3.1×10^{10} cells L^{-1} at d 8 following inoculation, and then decreased for the remaining period of incubation (Fig 5.3a). TBC showed significant inverse correlations with the concentrations of TDCHO of the HMW DOM ($r = -0.853$, $p < 0.01$) and LMW DOM ($r = -0.850$, $p < 0.01$). As observed for other parameters, BP rates were consistently higher in LMW-DOM than HMW DOM (Fig. 5.3b). Bacterial carbon accounted for 5.78 to 9.37 % of HMW DOC, and 7.96 to 21.26 % of LMW DOC (Fig. 5.3c). The concentrations of dissolved oxygen decreased in both the experiments over the course of 8 days, and there were significant differences in oxygen utilization between LMW and HMW incubations (Fig.5.3d). Rates of bacterial activity were greater for the LMW DOM than the HMW DOM (Table 5.5). In contrast, BGE was higher for HMW DOM than LMW DOM when DOC or TDCHO plus DURA were used to calculate BGE (Table 5.5).

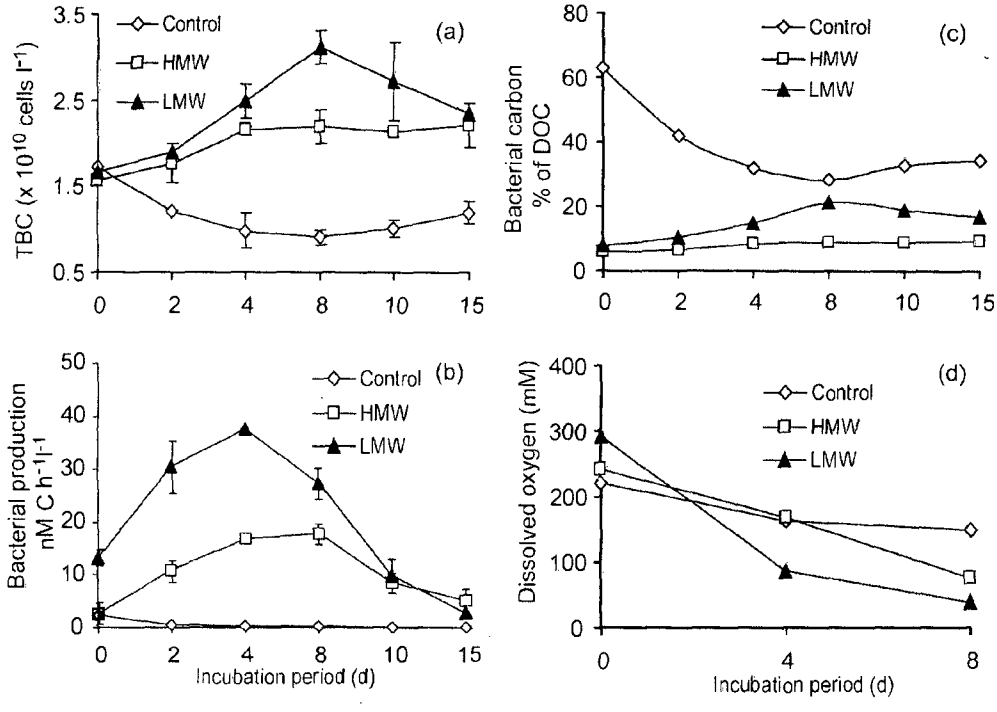


Fig. 5.3. Changes in bacterial abundance (a), bacterial production (b), changes in bacterial carbon % of DOC (c) and dissolved oxygen concentration (d) during HMW and LMW DOM degradation by natural bacterial population.

Table 5.5. Bacterial biomass (BB) (μg C $l^{-1}h^{-1}$), respiration rates (μg C $l^{-1}h^{-1}$), bacterial production (BP) (μg C $l^{-1}h^{-1}$), and bacterial growth efficiency (BGE) (%) in HMW and LMW fractions of organic matter.

Incubation	BB		Respiration rate		BP		BGE[a] (%)		BGE[b] (%)	
period (d)	HMW	LMW	HMW	LMW	HMW	LMW	HMW	LMW	HMW	LMW
0	0.00	0.00	-	-	0.03	0.16	0.00	0.00	0.00	0.00
2	0.63	0.75	-	-	0.13	0.37	4.33	3.65	27.44	5.39
4	0.91	1.31	4.51*	12.55*	0.20	0.45	6.01	6.80	26..00	8.42
8	0.49	1.14	5.14*	7.79*	0.22	0.33	9.06	6.84	29.03	10.63
10	0.35	0.67	-	-	0.10	0.12	4.88	2.95	15.12	4.32
15	0.27	0.29	-	-	0.06	0.04	4.32	1.17	6.78	1.91

BP was estimated by the [^{3}H] thymidine incorporation method using [^{3}H] thymidine conversion factor of 2×10^{18} cells per mol of [^{3}H] thymidine (Ducklow and Carlson, 1992). Bacterial cell numbers were converted to bacterial carbon using a conversion factor of 15 fgc per cell (Coron et al., 1995).

*= respiration was measured only on days 4 and 8.

BGE[a] = BP/change in DOC ×100. BGE was calculated from the DOC data given in table 5.2.

BGE[b] = BP/change in (TDCHO-C+DURA-C) ×100. BGE was calculated using the TDCHO and DURA data given in table 5.2.

5.4 Discussion

5.4.1 Source and reactivity of organic matter

Both LMW and HMW DOM fractions were utilized by natural bacterial populations. However, TBC and BP were relatively higher on LMW DOC (35%) than HMW DOC (13%) over the 15 d incubation period. This suggests that LMW DOM was more bio-reactive, and was preferentially used by bacteria. The difference in the relative reactivity of LMW DOM (>10 to 30 kDa) and HMW DOM (> 30 kDa to 0.22 µm) may be related to its source, size and TDCHO content. $\delta^{13}C$ values varied from - 27.0 to -26.2 ‰, and from -23.7 to -21.8 ‰ for HMW and LMW DOM size fractions, respectively (Table 5.2). $\delta^{13}C$ values for DOM derived from terrestrial sources or C3 plants varied from -28 to -26 ‰ (Raymond and Bauer, 2001; Cai et al., 2008). These values are similar to those observed for HMW DOM of the present study, suggesting that it was derived from terrestrial sources. In contrast, $\delta^{13}C$ values of LMW DOM (-23.7 to -21.8 ‰) are in the range of values reported for marine plankton (Raymond and Bauer, 2001; McCallister et al., 2004; 2006). Similarity in $\delta^{13}C$ values of LMW DOM and marine plankton suggest that it was derived from marine plankton. During the period of incubation, $\delta^{13}C$ values of LMW DOM were depleted. DOM is composed of heterogeneous mixture of organic compounds having different $\delta^{13}C$ values. Organic compound such as polysaccharides and proteins are enriched in $\delta^{13}C$ than lipids (Marchand, 2005; Teece et al., 2007). Selective removal of these compounds may account for the observed depletion of $\delta^{13}C$ of the LMW DOM. Subsequent, enrichment in $\delta^{13}C$ at day 10 and 15 probably indicates the production of

new organic compounds enriched in $\delta^{13}C$ (Marchand, 2005, Teece et al., 2007). This conclusion was supported by the increase in DPCHO concentration on d 15 of the incubation period (Table 5.2).

The ratio TDCHO-C/DOC is a useful indicator to assess the lability or nature of DOM (Cowie and Hedges, 1994; Kaiser and Benner, 2009). Freshly produced DOM is rich in reactive or labile components such as neutral carbohydrates (Cowie and Hedges, 1994; Meon and Kirchman, 2001; Kaiser and Benner, 2009). The fraction of DOC as carbohydrates was higher (25%) for LMW DOM and lower for HMW DOM (16%) at the start of the experiment (Fig. 5.2a). TDCHO contribution to DOC decreased rapidly for LMW DOM (from 25 to 9%), and slowly for the HMW DOM (16 to 9.0 %) over the period of incubation (Fig. 5.2a). The removal of TDCHO was higher (~75 %) for LMW DOM (>10 kDa to 30 kDa) and lower (46%) for HMW DOM (>30 kDa to 0.2 µm). The hypothesis that the HMW DOM is more terrestrial, and therefore less labile was indicated by smaller changes in TDCHO.

Our results indicate that TDCHO's are important substrates for the growth of planktonic heterotrophic bacteria, which is in agreement with previous studies (Hanisch et al., 1996; Kaiser and Benner, 2009) (Table 5.3). As the bacterial cell numbers increased, TDCHO concentrations decreased and MCHO concentrations increased. Similar observations have been recorded for marine (Burney, 1986; Cherrier and Bauer, 2004) and mesotrophic lake waters (Hanisch et al., 1996). This is also supported by higher MCHO/TDCHO ratio in LMW as compared to HMW DOM. The TDCHO utilization rates for the LMW DOM varied from 1.63 to 6.75 µg C l^{-}

$^{1}h^{-1}$ and were much higher than those recorded for HMW DOM (0.05 to 1.13 µg C $l^{-1}h^{-1}$). Our TDCHO utilization rates of LMW DOM also are higher than those recorded for other environments. For example, Burney (1986) reported that TDCHO utilization rate was 1.5 µg C $l^{-1}h^{-1}$ for the waters of the Atlantic. Jorgensen and Jensen (1994) observed a use rate of TDCHO of 2.75 µg C $l^{-1}h^{-1}$ for a mesotrophic Danish lake., TDCHO utilization rates varied seasonally (0.47 to 3.43 µg C $l^{-1}h^{-1}$) for mesotrophic lake Constance (Hanisch et al., 1996). Studies on the hydrolysis of polysaccharides using fluorogenic substrate analogs for glucosidase activity also indicate that TDCHOs are important substrate for growth of planktonic bacteria. Polysaccharides need to be hydrolysed to oligo-and monosaccharides before bacterial uptake. Chrost et al. (1989) observed good correlation between the concentrations of TDCHO and the activity of β-glucosidae during phytoplankton bloom in eutrophic lake Plußsee.

DURAs are acidic polysaccharides and generally account for a small proportion of organic carbon. DURA concentration in both HMW and LMW fractions decreased over the period of incubation implying their utilization by heterotrophic bacteria. DURA concentration decreased with increasing sediment core depth in the Dabob Bay (Bergamaschi et al., 1999), and with increasing water column depth in the Bay of Bengal (Khodse et al., 2007). In both the studies, the decrease in DURA concentration was suggested to be due to heterotrophic bacterial uptake. The utilization of DURA was greater for LMW DOM than HMW DOM implying that the former was utilized faster (Table 5.3, 5.4). The increase in DURA/TDCHO ratio for HMW DOM (0.46 to 0.76) and LMW DOM (0.32

to 0.96), as well as its significant positive correlations with TBC for both LMW DOM ($r = 0.949$, $p<0.001$) and HMW DOM ($r = 0.798$, $p<0.02$) (Fig. 5.2d) implies that TDCHO was preferentially utilized by bacteria as compared to DURA (Hung et al., 2001; Khodse et al., 2007).

5.4.2 Effect LMW and HMW DOM on TBC, BP, and BR

The higher TBC, BP and BR in LMW DOM than HMW DOM implies that the former DOM fraction was more bioreactive and preferentially utilized by natural marine bacterial population (Table 5.5). The $\delta^{13}C$ signature suggests that the LMW DOM was originated from marine phytoplankton, whereas HMW DOM was of terrestrial origin. The higher TBC, BP and BR in LMW DOM than HMW DOM suggest that bacteria preferentially assimilated an isotopically enriched $\delta^{13}C$ organic component derived from $\delta^{13}C$ enriched phytoplankton. Although we have a small dataset, our results support the preferential selection of algal material by heterotrophic bacteria despite the quantitative abundance of allochthonous organic matter (McCallister et al., 2006).

In oceanic waters dominated by algal production, HMW DOM (>1 kDa) as compared to the LMW DOM (<1kDa), is preferentially consumed by bacteria (Amon and Benner, 1994, 1996). In contrast, in riverine systems dominated by terrestrial inputs, LMW DOM (<1 kDa) is preferentially utilized by bacteria (Meyers et al., 1987; Volk et al., 1997; Covert and Moran, 2001; Axamanova et al., 2006). As compared to these contrasting views, Rochelle-Newall et al. (2004) reported that bioavailability of DOM is not constant and varied seasonally. Their results

demonstrated the flexibility of bacteria in their ability to utilize different sources of DOM, and highlight the variability that can be observed when different indices are used to determine the bioavailability of DOM to heterotrophic bacteria. From our results it is evident that nature and source of DOM are important in controlling utilization of DOM by bacteria.

Bacterial growth efficiency (BGE) is an important factor in understanding bacterial influence on carbon flow in aquatic ecosystems. BGE was greater in HMW DOM than LMW DOM. The higher BGE in the HMW DOM fraction indicate that the available HMW substrates are richer in organic nitrogen. This suggest that available portion of HMW DOM presumably has a lower C:N ratio than that of the reactive fraction of LMW DOM (Amon and Benner, 1996). Kroer (1993) observed an inverse relationship between BGE and the C/N ratio. He also reported that BGE increased from 18 to 37% due to addition of ammonium in the growth medium. Benner et al. (1988) also found that bacterial C conversion efficiencies on lignocellulosic substrates in fresh and estuarine water increased from 30 to 45% upon addition of ammonium and phosphate. Presumably some of these factors may be responsible for the higher BGE values in HMW DOM. According to Amon and Benner (1996) low growth efficiencies do not necessarily indicate that a certain DOC fraction is less bioreactive.

We have collected water sample from the Dona Paula Bay during the monsoon season when the Bay is strongly influenced by allochthonous inputs from the Mandovi and Zuari estuaries fringed with extensive mangroves. A high runoff of relatively easily degradable organic matter

and recalcitrant matter from terrestrial sources comes into the Bay during the south west monsoon. Wafer et al. (1997) estimated that 876 mg C m^{-2} d^{-1} of DOC of mangrove origin enters the Bay. It is likely that these features represent a large source of DOM in the Bay. This appears to be true because HMW DOM was derived from terrestrial sources, and had relatively lower TCHO-C/DOC ratio (Fig. 5.2a). TBC, BP and BR were relatively low in HMW DOM presumably because it was derived from terrestrial sources and was less labile due poor concentrations of TDCHO. Furthermore, the variability in bacterial utilization of LMW and HMW DOM fractions may also result from differences in the heterotrophic bacterial species or groups developed during the period of incubation (Covert and Moran, 2001; Cherrier and Bauer, 2004).

5.5 Conclusions

Bacterial utilization of HMW DOM (>30 t0 0.22 µm) and LMW DOM (>10kDa to 30 kDa) isolated from Dona Paula Bay, west coast of India was evaluated. $\delta^{13}C$ values suggest that HMW DOM fraction was mostly derived from terrestrial sources, whereas LMW DOM fraction was originated from marine phytoplankton. Moreover, DOM in LMW fraction contained higher TDCHO than the HMW DOM implying that the former DOM fraction was more labile. Bacteria preferentially utilised DOC, TDCHO and DURA of the LMW DOM as compared to DOC, TDCHO and DURA of the HMW DOM This conclusion was well supported by higher TBC, BP, BR and greater decrease in DOC, TDCHO and DURA in LMW DOM. Our results showed that bacterial consumption of size fractionated

DOM of the Dona Paula Bay was strongly affected by the source and nature of DOM.

Chapter 6

Summary

Summary

- In marine environments, carbohydrates are the most abundant and well characterized component of organic matter. However, most of the studies on the dynamics, production, distribution and cycling of carbohydrates are carried out in the temperate, Arctic and Antarctic water. In contrast, little is known about carbohydrates in tropical waters. In order to fill in this gap in our knowledge some aspects of carbohydrate studies were addressed during this research which showed some new and novel findings.
- Production of carbohydrate and uronic acid by four marine bacterial cultures (*Bacillus* spp.) and a marine fouling diatom *Amphora rostrata* was evaluated. Bacterial cultures were identified using morphological, biochemical and physiological characteristics and 16S rDNA technique. The effect of growth condition on the production of some carbohydrates by *Bacillus subtilis*, *Bacillus sp.*, *Bacillus licheniformis* and *Bacillus pumilus* was assessed.
- The concentration of substrate i.e. glucose rapidly decreased over the incubation period of bacteria. For all the bacterial cultures, concentrations of DPCHO and DURA increased over the period of incubation, and were generally greater during the stationary growth phase. EPSs produced by these bacterial cultures were isolated (>10 kDa), partially purified, and chemically characterized using standard analytical methods. Glucose and galactose were most abundant monosaccharides in the EPS produced by these bacteria.

The presence of uronic acid as well as several monosaccharides implies that the isolated polysaccharides were acidic heteropolysaccharides.

- *Amphora rostrata* is one of the most abundant diatoms in estuarine and marine waters. Therefore, carbohydrate production by *Amphora rostrata,* was evaluated. *Amphora rostrata* culture was grown in batch culture in f/2 medium for 10 days. During the cultivation period, chlorophyll *a*, TDCHO, MCHO, DPCHO and DURA were monitored over the period of cultivation. The concentrations of TDCHO, MCHO, DPCHO and DURA increased over the period of cultivation. During the cultivation period *Amphora rostrata* produced polysaccharides and uronic acids that accounted for 79% and 62% of the TDCHO, respectively. Fucose concentration increased over the period of cultivation. This implies that *Amphora rostrata* produced fucose rich polysaccharides during the period of cultivation.

- In aquatic environments, diatoms are found in attached (Biofilms) and planktonic growth modes. While attaching to the surfaces diatoms produce carbohydrates. Production and chemical characterization of carbohydrates in planktonic and biofilm cells of diatoms were evaluated. In order to identify, the differences in these two growth modes, we grew *Amphora rostrata* in batch cultures. Both planktonic (suspended in the growth medium) and biofilm cells (those attached to culture flasks) were analyzed for the total carbohydrate, carbohydrates fractions, neutral carbohydrates,

uronic acids, amino sugars, Chl *a* and cell abundance. These carbohydrate components were found to be different in planktonic and biofilm cells. Cell normalized concentrations of these biochemical components were 2 to 5 times higher in the planktonic as compared to biofilm cells of *Amphora rostrata*. Concentrations of glucose and glucosamine decreased, whereas, fucose increased in planktonic cells. In contrast, concentration of glucose and glucosamine increased while that of fucose decreased in attached cells.

- Carbohydrates are most abundant and well characterized components of dissolved organic matter. They are bioactive compounds and are utilized by heterotrophic bacteria as a carbon source. As a result, abundance of these compounds in aquatic environments influence ecosystem functioning. Therefore, in order to understand the dynamics of dissolved carbohydrates, such as monosaccharides (MCHO), total dissolved carbohydrates (TDCHO), HCl-hydrolysed dissolved carbohydrates (HCl-TDCHO), dissolved polysaccharides (DPCHO), HCl-hydrolysed polysaccharides (HCl-DPCHO), HCl-resistant polysaccharides (HR-DPCHO) and dissolved uronic acids (DURA), dissolved organic carbon (DOC) and a number of physical, chemical and biological parameters were monitored at several stations in the Mandovi estuary during the monsoon and pre-monsoon seasons. Phytoplankton (as Chl *a* and cell count) and bacterial abundance were higher during the pre-monsoon as compared to the monsoon season. Concentrations of

TDCHO, DPCHO and DURA showed strong seasonal and spatial variations. TDCHO and DURA concentration ranged from ~ 18 to 67 μM C, and 2.3 to 10.8 μM C, and their contribution to DOC varied from ~11 to 60% and 1.8 to 5.3%, respectively. DPCHO was a major fraction accounting for 52 to 92% of the TDCHO. Among the polysaccharides fractions, HR-DPCHO was the most abundant as they contributed 35 to 87% to the DPCHO. Generally, concentrations of various dissolved carbohydrate species and their contribution to the DOC was greater in monsoon. Bacterial abundance, bacterial production, phytoplankton abundance and nutrient concentrations influenced the distribution of carbohydrate including uronic acids in the pre-monsoon season but not during the monsoon season. In the Mandovi estuary, role of bacteria in controlling the distribution of various carbohydrates was evident from negative correlations between bacterial cell abundance and concentrations of various carbohydrate species.

- In another study from the Mandovi estuary, dynamics of particulate organic matter, especially particulate carbohydrates and particulate uronic acids was assessed. For this purpose, suspended particulate organic matter (SPM) was collected from various stations in the Mandovi estuary during the monsoon and the pre-monsoon seasons. The strong seasonal changes were observed for SPM, water discharge, POC, TPN content, $\delta^{13}C$ and $\delta^{15}N$. In the surface waters, concentrations of POC, TPN, TPCHO, TNPCHO and TPURA were influenced by the abundance of phytoplankton and

bacteria. Greater carbohydrate yields were associated with low Chl *a* and high POC/TPN ratio during monsoon indicating the presence of terrestrial organic matter during the monsoon season. This conclusion was also supported by the presence of depleted $\delta^{13}C$ values ($\delta^{13}C$ = -28.4) during the monsoon season. A number of monosaccharides arabinose (Arb), fucose (Fuc), galactose (Gal), glucose (Glu), mannose (Man), rhamnose (Rham), ribose (rib), xylose (Xyl) were identified in the SPM samples using gas chromatography. As compared to the monsoon, glucose abundance was relatively high during the pre-monsoon season implying that organic matter was relatively less degraded or fresh during this period. Higher Rham + Fuc mol % were associated with lower glucose content during monsoon implying that organic matter was degraded by bacteria. Carbohydrates showed significant positive correlation with the concentrations of Chl *a*. A negative correlation between carbohydrate yields and bacterial numbers suggested that bacteria played a major role in degradation of carbohydrates in the Mandovi estuary.

- In yet another study, distribution of particulate amino sugars was assessed to understand sources and degradation of organic matter in the Mandovi estuary. Amino sugar concentrations showed spatial and seasonal variation. Glucosamine (GlcN) and galactosamine (GalN) are major components of prokaryotic cell wall. GlcN is the main component of chitin polymer specially found in zooplankton. Low GlcN/GalN ratio (0.6 to 2.9) of the SPM samples indicates that

the POM was influenced by bacteria. Peptidioglycan is the only well known source for muramic acid (Mur), and hence is the specific biomarker for bacterial biomass. GlcN/Mur ratio of SPM samples varied from 2.1 to 17.2 and 3.0 to 22.8 during the monsoon and the pre-monsoon, respectively. The higher GlcN/Mur ratio was associated with low bacterial cell numbers and high Chl *a*, suggesting that GlcN concentrations were controlled by phytoplankton abundance. The muramic acid concentration showed significant positive correlation ($r = 0.602$; $p < 0.01$) with total bacterial numbers indicating that bacteria are major source of Mur. Mur can be used as a bacterial biomarker for marine samples. Based on the amino sugar ratios it is evident that amino sugars of SPM were derived from bacteria and phytoplankton. The glucosamine:muramic acid ratio (2.4 to 26.6) suggest that live bacterial cells are minor contributor of SPM. Bacterial carbon accounted for 4.7 to 11.9% and 5.9 to 14.6% of POC during the monsoon and the pre-monsoon season, respectively. Whereas, bacterial nitrogen accounted for 4.5 to 24.6% of TPN and 5.5 to 12.3% of TPN in the monsoon and the pre-monsoon season, respectively.

- DOM was collected from a station in the Dona Paula Bay, west coast of India, and fractionated into high molecular weight (HMW) (>30 kDa to 0.22 µm), and low molecular weight (LMW) (>10 kDa to 30 kDa) size fractions. Each size fraction was inoculated with natural bacterial population, and incubated over a period of 15 days

at room temperature (28 ±2 °C). During the period of incubation, sub-samples were removed and dissolved organic carbon (DOC), carbohydrate (TDCHO), monosaccharide (MCHO), uronic acid (DURA), $\delta^{13}C$, total bacterial count (TBC), bacterial production (BP), and oxygen consumption were measured.

- In this study, the question we asked was weather there were differences in the chemical and biochemical composition of HMW DOM fractions, and weather these differences influenced their utilization by natural bacterial populations. The LMW DOM fraction was isotopically heavier ($\delta^{13}C$ = -23.7 to -21.8 ‰), than the HMW DOM fraction ($\delta^{13}C$= -27.0 to -26.2 ‰), and the initial TDCHO content of these fractions were 25% and 16%, respectively. Uronic acids content was similar in LMW DOM (7.4 % OC), and HMW DOM (7.0 % OC). TBC, BP and bacterial respiration (BR) were consistently higher in LMW DOM than HMW DOM. Greater proportions of DOC (34 %), TDCHO (76 %), and DURA (36 %) of the LMW DOM fraction were utilized over the period of incubation than in HMW DOM. $\delta^{13}C$ values suggest that HMW DOM fraction was mostly derived from terrestrial sources, whereas LMW DOM fraction was originated from marine phytoplankton. Moreover, DOM in LMW fraction contained higher TDCHO than the HMW DOM implying that the former DOM fraction was more labile. Bacteria preferentially utilised DOC, TDCHO and DURA of the LMW DOM as compared to DOC, TDCHO and DURA of the HMW DOM This conclusion was well supported by higher TBC, BP, BR and greater

decrease in DOC, TDCHO and DURA in LMW DOM. This suggests that LMW DOM was most biologically reactive, less diagenetically altered, and hence it was more rapidly utilized than the HMW DOM. It appears that the nature and source of dissolved organic matter strongly influenced its utilization by natural heterotrophic bacteria.

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Appendix-I

a) Chemical composition of basal salt solution (BSS),

Chemical composition	Concentration
Sodium chloride (NaCl)	25.0 gm l^{-1}
Potassium chloride (KCl)	0.75 gm l^{-1}
Magnesium sulphate ($MgSO_4$. $5H_2O$)	7.0 gm l^{-1}
Ammonium chloride (NH_4Cl)	1.0 gm l^{-1}
K_2HPO_4 (10%)	7 ml
KH_2PO_4 (10%)	3 ml
Trace metal solution (TMS*)	1 ml
Distilled water	1000 ml
pH	7.5

b) Trace metal solution (TMS*) composition.

Chemical composition	Concentration
H_3BO_3	2.85 gm l^{-1}
$MnCl_2.4H_2O$	1.80 gm l^{-1}
$FeSO_4$. $7H_2O$	2.49 gm l^{-1}
Sodium tartarate	1.77 gm l^{-1}
$CuCl_2$	0.0269 gm l^{-1}
$ZnCl_2$	0.0208 gm l^{-1}
$CoCl_2$	0.0404 gm l^{-1}
$Na_2MoO_4.2H_2O$	0.0253 gm l^{-1}

Appendix-II

Composition of Guillard and Ryther's f/2 medium for the culturing of diatoms (1962):

Sr. No.	Chemical	Concentration
	Stock solution I	
1	Sodium nitrate ($NaNO_3$)	7.5 gm
2	Sodium dihydrogen phosphate (NaH_2PO_4. H_2O)	0.5 gm
3	Sodium Silicate (Na_2SiO_3. $9H_2O$)	1.5 to 3.0 gm
4	Milli-Q water	100 ml
	Stock solution IIa (100 ml)	
5	Copper sulphate ($CuSO_4$. $5H_2O$)	0.98 gm
6	Zink sulphate ($ZnSO_4$. $7H_2O$)	2.20 gm
7	Cobaltous chloride ($CoCl_2$. $5H_2O$)	1.00 gm
8	Manganous chloride ($MnCl_2$. $4H_2O$)	18.0 gm
9	Disodium molybdate (Na_2MoO_4. $2H_2O$)	0.63 gm
	Stock solution IIb (1000 ml)	
10	Ferrous chloride (FeCl. $6H_2O$)	3.15 gm
11	EDTA-sodium salt	4.36 gm
	Stock solution III (vitamins)	
12	Thiamin HCl	20.0 mg
13	Biotin (primary stock solution)	1.00 gm
14	Vitamin B_{12}	0.1 ml
15	Milli-Q water	100 ml

The stock solutions were added to 1000 ml of aged seawater as follows:

Stock solution I - 1 ml

Stock solution II* - 1 ml

Stock solution III - 0.5 ml

Aged seawater - 997.5 ml

Note:

1) During preparation of stock solution II, all the metal solutions of stock solution IIa were prepared separately in 100 ml Milli-Q water. The constituents of stock solution IIb were dissolved in 900 ml Milli-Q water to which 1 ml of each metal solutions of stock solution IIa was added. The final volume was made to 1000 ml.

2) To minimize the precipitation of salts on autoclaving, the silicates and phosphates were sterilized separately and added after autoclaving the seawater.

3) Stock solution of vitamins was filter sterilized before adding to the sterilized medium.

List of published papers

1. Vishwas B. Khodse, Narayan B. Bhosle, S.G. Prabhu Matondkar. 2010. Distribution of dissolved carbohydrates and uronic acids in a tropical estuary, India. Journal of Earth System Science, 119, 1-12. (**IF = 0.8**)

2. Vishwas B. Khodse and Narayan B. Bhosle 2010. Differences in carbohydrate profiles in batch culture grown planktonic and biofilm cells of *Amphora rostrata Wm.Sm.* Biofouling, 26, 527-537. **(IF = 4.4)**

3. Vishwas B. Khodse, Narayan B. Bhosle, V.V. Gopalkrishna. 2009. Distribution of particulate carbohydrate species in the Bay of Bengal. Journal of Earth System Science, 118 (2), 147-156. **(IF = 0. 8)**

4. Vishwas B. Khodse, Loreta Fernandes, Narayan B. Bhosle, Sugandha Sardessai. 2008. Carbohydrates, uronic acids and alkali extractable carbohydrates in contrasting marine sediments: distribution, size fractionation and partial chemical characterization. Organic Geochemistry, 39, 265-283. **(IF = 2.3)**

5. Vishwas B. Khodse, Loreta Fernandes, V.V. Gopalkrishna , Narayan B. Bhosle, Veronica Fernandes, S.G. Prabhu Matondkar, Ravi Bhushan. 2007. Distribution and seasonal variation of concentrations of particulate carbohydrates and uronic acids in the northern Indian Ocean. Marine Chemistry, Vol. 103, 127-146. **(IF= 2.9)**

6. Vishwas B Khodse and Narayan B Bhosle. Bacterial utilization of size fractionated dissolved organic matter. Aquatic Microbial Ecology. 2011, 64, 299-309.

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Bacterial utilization of size-fractionated dissolved organic matter

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ABSTRACT: Dissolved organic matter (DOM) is an important source of organic carbon for sustaining the growth of heterotrophic bacteria. We investigated the bacterial utilization of high-molecular-weight (HMW; >30 kDa to 0.22 µm) and low-molecular-weight (LMW; >10 to 30 kDa) fractions of DOM. DOM was collected from a station in Dona Paula Bay, on the west coast of India, and fractionated into HMW and LMW portions. Each size fraction was inoculated with a natural population of bacteria and incubated over a period of 15 d at room temperature (28 ± 2°C); during this period, sub-samples were removed and the following were measured: dissolved organic carbon (DOC), total dissolved carbohydrate (TDCHO), free monosaccharide (MCHO), dissolved uronic acid (DURA), $\delta^{13}C$, bacterial abundance (BA), and bacterial production (BP). The LMW fraction was isotopically heavier ($\delta^{13}C$ = −23.7 to −21.7‰) than the HMW fraction ($\delta^{13}C$ = −27.0 to −26.2‰), and the initial TDCHO content of these fractions was 25 and 16%, respectively. The initial DURA content was similar in the LMW DOM (7.4% DOC) and the HMW DOM (7.0% DOC). BA and BP were consistently higher in the LMW DOM than in the HMW DOM. In 15 d incubation, greater proportions of DOC (35%), TDCHO (76%) and DURA (36%) were utilized in the LMW DOM than in the HMW DOM. This suggests that the LMW DOM was more biologically reactive, i.e. it was utilized more rapidly, than the HMW DOM. It appears that the bioreactivity and origin of the DOM strongly influenced its utilization by natural heterotrophic bacteria.

KEY WORDS: Dissolved organic matter · Size fractions · Carbohydrates · Polysaccharides · Bacteria · Bacterial production · Dona Paula Bay

INTRODUCTION

In the marine environment dissolved organic matter (DOM) stores a large amount of biologically reactive organic carbon that is equal to the amount of carbon dioxide (CO_2) in the earth's atmosphere (Hedges 1992). The sources (terrestrial or marine) and cycling of dissolved organic carbon (DOC) are of considerable importance in the global carbon cycle (Kirchman et al. 2009, Porcal et al. 2009). Phytoplankton primary production is the main source of DOC in marine environments, whereas allochthonous inputs can be important in freshwater systems (Gueguen et al. 2006). Heterotrophic bacteria are major consumers and remineralizers of DOC. In some ecosystems up to 40 to 60% of autochthonous primary production is cycled through bacteria (Hoch & Kirchman 1993). The availability of DOM to heterotrophic bacteria depends on factors such as biochemical composition, molecular size, inorganic nutrient concentrations, photochemical transformation, temperature, and the structure of the microbial community (Amon & Benner 1996, Carlson et al. 2004, Rosenstock et al. 2005, Abboudi et al. 2008).

Total dissolved carbohydrate (TDCHO) is one of the largest pools of bioreactive DOM in aquatic environments. TDCHO accounts for a significant fraction of the DOC pool in both oceanic waters (20 to 30%)

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Distribution of dissolved carbohydrates and uronic acids in a tropical estuary, India

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Carbohydrates including uronic acids are among the active components of dissolved organic carbon, and play an important role in biogeochemical cycling of organic carbon in marine environments. In order to understand their distribution, concentrations of total dissolved carbohydrate (TCHO), dissolved polysaccharide (PCHO), dissolved monosaccharide (MCHO), and dissolved uronic acid (URA) were measured in the Mandovi estuary, west coast of India during the monsoon and pre-monsoon seasons. The estuary experienced nearly fresh water condition during the monsoon season and marine condition during the pre-monsoon season. Concentrations of TCHO, MCHO and URA ranged from 17.7 to 67.3 μM C, 4.1 to 15.5 μM C and 2.3 to 10.8 μM C, and their contribution to dissolved organic carbon (DOC) varied from $\sim$11 to 60%, 2.5 to 9.7%, and 1.8 to 5.3%, respectively. PCHO accounted for $\sim$52 to 92% of the TCHO. Generally, concentrations and yields of TCHO species were greater during the monsoon season. Phytoplankton abundance and bacterial cell numbers influenced the distribution of TCHO in the pre-monsoon season but not during the monsoon season. Transport of TCHO rich (11 to 60%) dissolved organic matter from the Mandovi estuary to the coastal waters during the monsoon season may affect ecosystem function by fueling biological activity of heterotrophic micro-organisms.

1. Introduction

Carbohydrates are ubiquitous in marine environments, and are associated with biogeochemical processes taking place in marine environments (Bada and Lee 1977). Dissolved carbohydrates account for a large fraction ($\sim$10 to 85%) of dissolved organic carbon (DOC) in seawater (Romankevich 1984; Thurman 1985; Pakulski and Benner 1994; Hung *et al* 2001, 2005), pore waters (Burdige *et al* 2000) and ultrafiltered DOC (Amon and Benner 2003). Dissolved carbohydrates are derived from various processes including direct release by organisms (Biddanda and Benner 1997; Hama and Yanagi 2001), microbial degradation of organic matter (Hellebust 1965), viral or bacterial lysis of phytoplankton cells (Jumars *et al* 1989) and allochthonous DOC from rivers and soil (Gueguen *et al* 2006). Dissolved carbohydrates are useful substrates for heterotrophic bacteria (Rich *et al* 1997; Kirchman *et al* 2001). Heterotrophic uptake and secondary production influence the abundance of dissolved carbohydrates in aquatic environments (Satoh *et al* 1986; Meon and Kirchman 2001). Due to their reactive nature, carbohydrates have been proposed as indicators of the bioreactivity and diagenetic state of natural dissolved organic matter (Skoog and Benner 1997; Amon and Benner 2003). Distribution of dissolved total carbohydrate (TCHO), dissolved monosaccharide (MCHO), and polysaccharide (PCHO) has been reported from different environments including rivers (Hung *et al* 2005; Gueguen *et al* 2006; Cai *et al* 2008), estuaries (Senior and Chevolot 1991), coastal and oceanic waters (Burney and Sieburth 1977; Pakulski and Benner 1994; Bhosle *et al* 1998; Hung *et al* 2001,

Keywords. Carbohydrates; polysaccharides; uronic acids; phytoplankton; bacteria; tropical Mandovi estuary.

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Differences in carbohydrate profiles in batch culture grown planktonic and biofilm cells of *Amphora rostrata* Wm. Sm

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Diatoms are abundant in biofilms developed on surfaces immersed in sunlit waters. In both the planktonic and the biofilm mode of growth, diatoms produce carbohydrate polymers which perform several functions including motility, protection, production of macro-aggregates and detoxification. However, little is known about the differences, if any, in the production and characterization at the molecular level of carbohydrates in planktonic and biofilm cells. In order to identify the differences in these two modes of growth, the concentration of total carbohydrates, carbohydrate fractions, neutral carbohydrates, uronic acids and amino sugars in planktonic and biofilm cells of *Amphora rostrata* were measured. The results showed that the distribution of carbohydrate fractions, uronic acids and amino sugars was different in biofilm and planktonic cells. Cell normalized concentrations of these components were two to five times greater in planktonic cells compared with biofilm cells. The concentrations of glucose and glucosamine decreased, whereas fucose increased in planktonic cells over the period of cultivation. Conversely, the concentrations of glucose and glucosamine increased while that of fucose decreased in attached cells. The study suggests that marked differences exist between the carbohydrates of the planktonic and the biofilm cells of *A. rostrata*.

Keywords: carbohydrate; glucose; fucose; glucosamine; protein; planktonic cells; biofilm cells; *Amphora rostrata*

Introduction

Solid surfaces immersed in aquatic environments adsorb dissolved organic matter which conditions the surface and moderates colonization by various micro-organisms such as bacteria, algae, and fungi. Attached microorganisms enmeshed in exopolymeric substances are known as biofilms. Diatoms (unicellular algae) are amongst the earliest autotrophic colonizers of surfaces immersed in sunlit environments (D'Souza and Bhosle 2003; Mitbavkar and Anil 2008; Molino et al. 2009). Diatoms are also known to release extracellular organic compounds into the growth medium and in biofilms these may serve as carbon sources for heterotrophic bacteria (Bahulikar and Kroth 2008; Bellinger et al. 2009). Therefore the quality and quantity of extracellular organic compounds released by diatoms may influence the structure of the micro-fouling community (Decho 1990; Hoagland et al. 1993; Molino and Wetherbee 2008).

While attaching to surfaces, diatoms produce extracellular polymeric substances (EPS), which are rich in carbohydrates. Carbohydrates are common structural and storage products of both biofilm and planktonic diatoms (Cooksey and Wigglesworth-Cooksey 1995; Smith and Underwood 1998, 2000; Bellinger et al. 2009). The quality, quantity, and composition of these polymers are influenced by the nutrient status and growth phase (Myklestad 1977; D'Souza and Bhosle 2001). Several functions including protection and detoxification have been ascribed to diatom polymers. Carbohydrate polysaccharides bind to other organic molecules by various linkages which can be hydrolyzed using treatment with heat or alkali or both (Handa 1969; Haug and Myklestad 1976; Hitchcock 1977). Therefore, polysaccharides can be isolated using a selective sequential extraction technique (Handa 1969; Haug and Myklestad 1976; Hitchcock 1977; Bhosle et al. 1993).

When grown under normal experimental conditions, some diatom cells attach to culture flasks and form biofilms while others remain suspended in the culture medium. The diatom cells living within a biofilm may have a different carbohydrate profile compared with those of free-floating (planktonic) forms. Understanding the biochemistry of the polymers produced by diatoms under different growth conditions is important to enhance knowledge regarding the processes that lead to biofilm development, or why some organisms maintain a planktonic lifestyle. However, little is known about the differences, if any, in the cellular carbohydrates in attached and planktonic cultures of diatoms because earlier studies have mostly focused on the characterization of extracellular polysaccharides

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Distribution of particulate carbohydrate species in the Bay of Bengal

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Suspended particulate matter (SPM) of surface seawaters was collected during December 2003 to October 2004 at 10 stations in the Bay of Bengal, and analyzed for particulate organic carbon (POC), total particulate nitrogen (TPN), total particulate carbohydrate (TPCHO) and total particulate uronic acids (TPURA). The concentrations of POC, TPCHO and TPURA varied from 4.80 to 29.12, 0.85 to 4.24, 0.09 to 0.91 μM C, respectively. The TPCHO-C and TPURA-C accounted for 6.6–32.5% and 0.87–3.65% of POC. The trends observed for the distribution of these compounds were generally similar to those recorded for the distribution of chlorophyll *a* (Chl *a*). The C/N ratios varied from 3.2 to 22.3 with most of the values being < 10. This suggests that the organic matter was mostly derived from phytoplankton and bacteria. Relatively low C/N ratios and high TPCHO yield imply that freshly derived organic matter was present during SWM and FIM. Our data suggest that the quality and quantity of organic matter varied spatially and seasonally.

1. Introduction

Carbohydrates are the most important constituents produced by phytoplankton during the process of photosynthesis. Typically, carbohydrates comprise more than 40 wt% of bacteria and 75 wt% of vascular plants (Moers and Larter 1993), 20–40 wt% of plankton (Parsons *et al* 1984b), 13% of sinking particulate organic carbon (Tanoue and Handa 1987), ~ 3–35% of suspended particulate organic carbon (Khodse *et al* 2007), 10–85% in seawater dissolved organic carbon (DOC) (Pakulski and Benner 1994) and sediment pore water DOC (Burdige *et al* 2000), and 3–26% of sedimentary organic carbon (Khodse *et al* 2008). These compounds are useful substrates for heterotrophic organisms. It appears that the concentration and composition of carbohydrates are influenced by several factors including nutrient levels, composition of organisms, growth phase, etc. Carbohydrates are classified as structural and storage components of both marine and terrestrial organisms. The storage carbohydrates are utilized preferentially. This results in the accumulation of relatively less degradable structural carbohydrates in the degraded organic matter.

In addition to neutral carbohydrates, many marine micro-organisms, terrestrial plants, algae and other organisms produce uronic acids or acidic carbohydrates (Bergamaschi *et al* 1999; Hung *et al* 2003). They are also present in dissolved organic matter, particulate matter, and marine sediments (Bergamaschi *et al* 1999; Hung *et al* 2003; Khodse *et al* 2007, 2008). Although present in small amounts, they are involved in many marine processes such as the production of humic substances and mucilaginous aggregates, biofilm formation and bacterial adhesion and detoxification of toxic chemicals and removal of metal ions (Decho 1990).

There are many studies describing the physical, chemical and biological characteristics of the

Keywords. Suspended particulate matter; chlorophyll *a*; particulate organic carbon; C/N ratio; particulate carbohydrates; particulate uronic acids; organic matter, Bay of Bengal.

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Carbohydrates, uronic acids and alkali extractable carbohydrates in contrasting marine and estuarine sediments: Distribution, size fractionation and partial chemical characterization

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Abstract

Concentration, size fractionation and monosaccharide composition of carbohydrates and uronic acids were investigated in contrasting sediments of the Mandovi estuary (ME), Arabian Sea (AS) and the Bay of Bengal (BOB). Concentrations and monosaccharide composition of carbohydrates and uronic acids varied spatially. Average yields of carbohydrates and uronic acids were higher for the estuarine compared to marine sediments. Interestingly, yields of carbohydrates and uronic acids increased in sediments with water column depth, implying preferential removal of other constituents, selective preservation and/or lateral input of carbohydrate rich material. Analysis of monosaccharide biomarkers indicates that carbohydrates were derived from terrestrial plants, bacteria and phytoplankton, however, the influence of the former was relatively greater in sediments of the ME and BOB, as well as in residual sediments of ME. Approximately 11–21% of total carbohydrates could be extracted using hot alkali extraction followed by sonication. Irrespective of the depositional environment, carbohydrates and uronic acids were greater in the very high molecular weight size fraction (>30 kDa). The abundance of glucose increased with the decrease in molecular size of extracted organic matter. This probably indicates its association with less degradable carbohydrates.

1. Introduction

In the biosphere, carbohydrates are the major organic compounds produced photosynthetically by autotrophic organisms. They account for a substantial amount of the dissolved and particulate organic carbon in the water and sediments of marine environments. For example, they may account for 10–85% of the dissolved organic carbon (DOC) in seawater (Pakulski and Benner, 1994) and sediment pore waters (Arnosti and Holmer, 1999; Burdige et al., 2000), 3–16% and 2–13% of the particulate organic carbon (POC) of the suspended (D'Souza and Bhosle, 2001), and sedimenting particles, respectively (Hernes et al., 1996; D'Souza et al., 2003; Panagiotopoulos and Sempere, 2005), and 3–20% in

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Distribution and seasonal variation of concentrations of particulate carbohydrates and uronic acids in the northern Indian Ocean

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Abstract

Suspended particulate matter (SPM) samples were collected from the surface seawaters at 31 stations, and from various depths (2 to 1000 m) at 9 locations in the northern Indian Ocean during various seasons. SPM samples were analyzed for total particulate carbohydrate (TPCHO), total particulate uronic acid (TPURA) and total particulate neutral carbohydrate (TPNCHO) concentrations and composition. Strong spatial, temporal and depth related variations were evident in the distribution of these compounds. In surface waters, concentrations of TPCHO, TPNCHO, and TPURA varied from 0.57 to 3.58 μM C, 0.11 to 2.34 μM C, and from 0.01 to 0.31 μM C, respectively, and accounted for 2.6 to 34.6%, 2 to 24.5%, and 0.2 to 6.3% of POC, respectively, whereas the TPURA accounted for 4.7 to 22.7% of TPCHO. Concentrations and yields of both TPNCHO and TPURA decreased rapidly in the upper 100 m of the water column suggesting their utilization by heterotrophic organisms. Glucose was the most abundant constituent of the TPNCHO. Glucose mole fraction decreased while that of other monosaccharides, especially galactose, arabinose, mannose, rhamnose and fucose increased in the upper 100 m water. Below this depth, mole fraction of glucose increased while that of other sugars decreased with the increasing water depth. Generally, high C:N ratios were associated with low yields of carbohydrates and uronic acids. Inverse correlation between the mole fractions of arabinose plus xylose and rhamnose plus fucose indicates the importance of biogenic and terrestrial organic matter input to the Bay of Bengal. TPURA are surface-active in nature and thus may play an important role in coagulation of particles and macromolecules. The observed spatial and seasonal variations of these compounds may be due to differences in phytoplankton biomass, nutrient status, and the influence of terrestrial material.

Keywords: Total carbohydrates; Neutral carbohydrates; Uronic acids; C:N ratio; POC:Chlorophyll *a*; Carbohydrate yields; Monosaccharides; Northern Indian Ocean

1. Introduction

In oceanic environments, particulate organic matter (POM) is mostly derived from phytoplankton (Deuser et al., 1981). Conversely, POM in the coastal and shelf waters is influenced by the inputs of terrestrial organic matter discharged by rivers and re-suspension of bottom sediments (Degens and Ittekkot, 1985). In aquatic environments, POM serve as an important source of food for the aquatic organisms. Moreover, POM is of considerable biogeochemical and oceanographic importance because it serves as a vehicle for the transport of

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