STUDIES ON SOME ASPECTS OF MARINE MICROBIAL EXOPOLYSACCHARIDES

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OF

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IN
MICROBIOLOGY

BY
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UNDER THE GUIDANCE
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APRIL 2003

CERTIFICATE

This is to certify that the thesis entitled **"Studies on some aspects of marine microbial exopolysaccharides"** submitted by **Mr. P. V. Bhaskar** 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.

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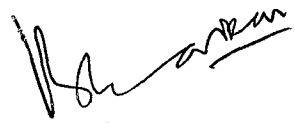
STATEMENT

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled **"Studies on some aspects of marine microbial exopolysaccharides"** 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.



(P. V. Bhaskar)

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P.V.BHASKAR

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Dedicated to my

Grandparents

CHAPTER 1

General Introduction

Chapter 1

1.1 Microbial exopolysaccharides (EPS)

1.1a What is EPS?

Most microorganisms produce varying amounts of extracellular organic polymers that are principally made of polysaccharides with smaller quantities of proteins, lipids, glycoproteins etc (Costerton et al 1981). Such extracellular polymers are called as exopolysaccharides or EPS (Wingender et al 1999). Microbial EPS is produced by both prokaryotes (eubacteria and archaebacteria) and eukaryotes (phytoplankton, fungi, yeasts, and algae) that form a gel-like matrix around the microorganisms. EPS is produced by microorganisms as free planktonic forms in aquatic environments (Burns 1989, Nielsen et al 1997, Jahn and Nielsen 1998), in association with other organisms (symbiotic existence) (Rosen and Cornford 1971, Costerton et al 1978) or when attached to natural and artificial surfaces (man-made structures) (Decho 1990, Vandivivere and Kirchman 1993, Becker 1996).

Microbial EPS are basically of two types- capsular and slime layer. The capsule is highly hydrated polymeric structure tightly enveloped around the cell and forms discrete extracellular structures (**Fig. 1.1**). These are held to the cell-wall either by linkages between the carboxyl groups of EPS and hydroxyl groups of lipopolysaccharides (LPS) (Sutherland 1977) or through a

covalent bonding through phospholipid (Roberts 1996) and glycoproteins (Chester and Murray 1978). On the other hand, slime layer is a less organized structure, which is loosely held to the cell surface and can easily diffuse into

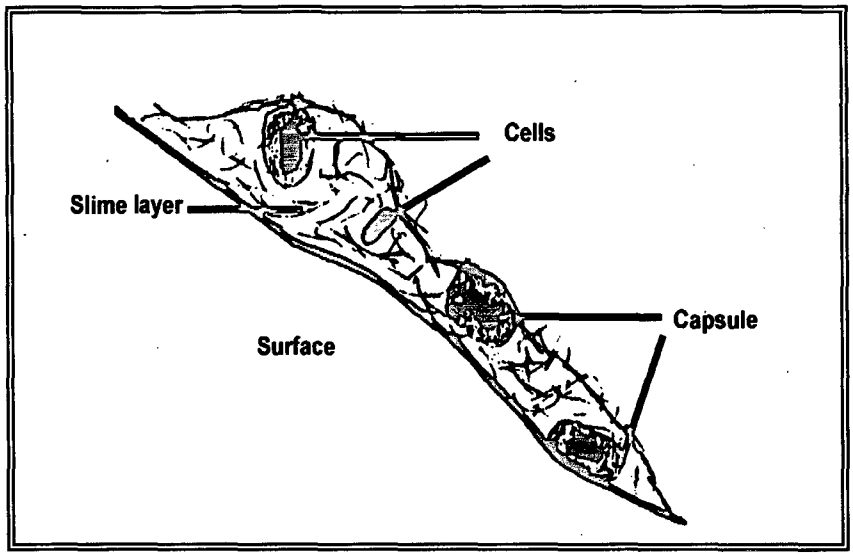

Fig. 1.1 Schematic representation of a biofilm showing the cells enveloped with a capsule and embedded in a matrix of slime EPS. (*Reproduced from Decho (1990)*)

the surrounding waters (Decho 1990) (**Fig. 1.1**). Sheaths are another forms of capsular EPS which envelope a chain of cells (Takeda et al 1998). EPS is highly hydrated polymer and contains up to 99% water. Therefore, EPS is invisible when viewed directly under microscope and need to be stained with either India ink, anion stains like ruthedium red and alcian blue. The electron microscopic images of both capsules and slimes of bacteria reveal a matrix of polysaccharide fibrils. These fibrils in the capsules are tightly wound around the cell-surface (Decho 1990). High-resolution cryo-freeze etching SEM (cryo-FESEM) techniques reveal the fine fibrillar composition of extracellular sheaths in stalked diatoms (Wang et al 2000).

1.1b Chemistry of EPS

Microbial EPS is composed of a long chain of sugars that form the backbone of the polymer. Most of these polymers are generally of high molecular weight polysaccharides (10 to 30 kDa) with variable composition. This chain might be unbranched or branched with side chains of other compounds attached to the polymeric chain (Sutherland 1977). Based upon the composition of the chain, the polymer is classified as homopolysaccharides (having the same monomer) or heteropolysaccharides (different repeating monomer). For example, bacterial cellulose, dextran (**Fig. 1.2a**), levans etc are some of the major homopolymers having glucose as the repeating unit (Sutherland 1983). On the other hand, EPS like bacterial alginate (**Fig. 1.2b**) and most other polymers are heteropolymers with more than two monosaccharides and other additional groups like uronic acid (Sutherland 1977). The monomers of the polysaccharide chain are joined by either α-D or β-D linkages. For example, exopolymers like pullulans are made of glucans linked by β-D (1,3 and 1,6) linkages (Harada 1977) whereas dextran is made of glucans linked by α-D (1,6; 1-4; 1-3 & 1-2) linkages (Jeanes 1978). The monosaccharide composition of the EPS also varies with the source. For example, bacterial EPS are generally rich in hexoses like glucose, galactose, rhamnose, mannose and fucose (Sutherland 1977) than pentoses like xylose (Powell 1979). Apart from monosaccharides, microbial EPS also have carboxylated sugars like uronic acids and non-sugar components like proteins, aminosugars, lipids, pyruvates, ester sulphates and nucleic acid. Generally, these components are present in relatively smaller quantities but are

important in regulating the characteristics of the microbial EPS (Decho 1990). Moreover their contribution to the total EPS varies considerably with the source. One of the major components of the

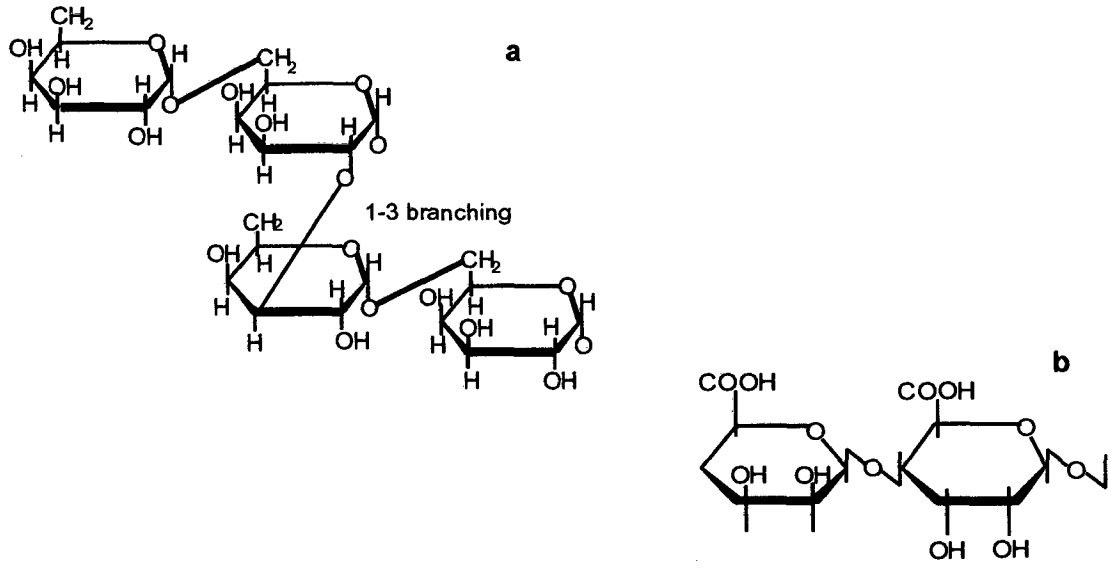

Fig. 1.2 Chemical structure of dextran, a homopolsaccharide made of α-1-6 glucan monomers with 1-3 branched linkages (a) and bacterial alginate, a heteropolysaccharide having a combination of D-mannuronic acid and L-guluronic acid linked by β-1,6 linkage.

EPS is uronic acid that may constitute up to 20-50% of the polysaccharide fraction in marine bacterial EPS (Kennedy and Sutherland 1987, Majumdar et al 1999). Other non-sugar components like phosphates, pyruvates and acyl groups present in the EPS are generally attached to the sugar residues (Sutherland 1979). The presence of pyruvates and acyl groups prevents the epimerisation of uronic acid, thereby ensuring higher content of uronic acid in the polymer (Decho 1990). The higher yield of uronic acid gives an overall negative charge to the polymer, thereby imparting adsorptive properties to the

polymer (Smidsrod 1974). Apart from organic non-sugar moieties, cations like Ca^{2+} and Mg^{2+} have also been widely reported in both bacterial and diatom EPS (Marshall et al 1971, Cooksey 1981). The presence of divalent cations helps in multiple cross-linkages between the sugar molecules of different polysaccharide chains, thereby imparting a high degree of consistency and stability to the EPS (Lewin 1956, Williams et al 1972, Decho and Moriarty 1990).

1.1c Regulation of EPS production

The production of EPS by microorganisms is influenced by various factors including the physiological state of the organism, age of the organism, nutrient levels and nutrient composition (Decho 1990). Bacteria and to a lesser extent phytoplankton produce exopolysaccharides throughout different stages of their life-cycle. However, maximum EPS production generally occurs at the end of the log-phase in most bacteria and phytoplankton species (Myklestad and Haug 1972, Mykelstad 1977, Williams and Wimpenny 1977 & 1978). In both natural environments and laboratory, microorganisms tend to produce more EPS under nitrogen limiting conditions. Increase in carbohydrate concentrations have been reported at the end of phytoplankton blooms in the oceanic environments (Ittekkot et al 1981), mesocosm studies (Mopper et al 1995) and laboratory studies (Henriques-Viera and Myklestad 1986, Fajon et al 1999), coinciding with depletion in nutrients like nitrate and phosphate. Similarly, bacteria also produce maximal EPS in the late log-phase under low nutrient conditions (Williams and Wimpenny 1977 & 1978). Moreover, bacteria

tend to produce more EPS when supplied with higher concentrations of carbohydrates as substrate (Sutherland 1972). Apart from higher concentrations of carbohydrate, the presence of precursors like acetyl CoA, isoprenoids, nucleoside diphosphates sugars and phosphoenol pyruvates have also been shown to enhance the production of EPS (Norval and Sutherland 1969, Sutherland 1979, Whitfield 1988). Other factors that influence the growth of the organisms like pH, aeration and temperature also regulate the production of EPS (Williams and Wimpenny 1977 & 1978). For marine organisms, variation in salinity can also lead to alteration in EPS yield (Allan et al 1972). The genetic regulation of EPS production affects the ability of the organism to produce EPS (Avery et al 1944). Fragments of DNA like transposons and insertion elements are considered important in regulating the production of EPS in some pathogenic bacteria (Bartlett et al 1988). The exact mechanism of genetic control over EPS production is however debatable.

1.1d Biosynthesis of EPS

The biosynthesis of EPS in bacteria can either take place 1) extracellular; utilizing the precursors from the extracellular environment or 2) intracellular, assembled near the membrane and then released outside (Decho 1990). In the extracellular synthesis, precursors like sucrose are used (Sutherland 1977) and the EPS thus produced are characteristically homopolysaccharides. In order to synthesize the polymer, cells produce extracellular transferase but do not require intermediates like nucleoside diphosphate sugars. On the other hand, intracellular production is more common and is found in most bacteria. This type of synthesis yields

heteropolysaccharides. In order to synthesize such polymers, activated precursors like nucleoside diphosphate sugars and carrier molecules like lipid intermediates are required (Norval and Sutherland 1969). The formation and partial assembly of the polymer take place close to cytoplasmic membrane and extruded out into the extracellular environment where further elongation takes place. The synthesis is independent of the substrate and physiological conditions. The site of EPS synthesis in majority cases is at the inner membrane (Osborn et al 1972). The EPS is transported into the external environment through porins located on the membrane (Sutherland 1977). In contrast to bacteria, EPS is in diatoms and microalgae are internally synthesized and contained in vesicles produced in Golgi apparatus (Daniel et al 1980). These vesicles are transported to the cell membrane and secreted through longitudinal channels called raphe (Daniel et al 1980).

1.1e Functions of EPS

EPS produced by the microorganisms perform various important functions that help the cells survive in extreme conditions. One of the most important functions of EPS is adhesion of cells to natural and artificial surfaces – animate or inanimate. During adhesion, microorganisms first come closer to the surface where they are held by weak Van der Waal forces of attraction. This is defined as the "reversible sorption" wherein organisms still exhibit Brownian motility and are easily removed from the surface by moderate shear forces (Marshall et al 1971). The reversible sorption depends upon factors like electrostatic forces, surface charges, hydrophobicity of the surface and the

availability of cations. Once the cells are held closer to the surface, they produce EPS that enables the cells to attach irreversibly to the surface and grow, thereby forming biofilms (Costerton et al 1981, Costerton 1984). The production of EPS during the formation of biofilms has many advantages including better access of microorganisms to nutrients, development of microbial consortia, horizontal migration of genetic material, protection against desiccation and toxicity etc (Decho 1990). In the field of medicine, production of capsular EPS can enhance the resistance of pathogens against host immune system (Deighton et al 1996, Pasquier et al 1997). In some microorganisms, the EPS released during the formation of biofilms can produce immunogenic response from the hosts. For example, the production of K-antigen by some *E. coli* serotypes is basically a capsular lipopolysaccharides (LPS) (Whitefield 1988). On the other hand, the formation of biofilms on artificial surfaces results in the conditioning of the surface, thereby serving as a cue for other organisms to colonize the surfaces (Szewzyk et al 1991). Such phenomena are commonly encountered in all submerged structures, affecting their strength and performance. Bacterial biofilms on metal surfaces are also involved in regulation of corrosion. Many bacterial EPS contain acidic moieties like uronic acids, phosphoric acid groups etc (Martin 1971). EPS has the ability to form complex with metal ions (Friedman and Dugal 1968, Martin et al 1972, Bitton and Friehoffer 1978, Brown and Lester 1982, Mittleman and Geesey 1985). Although EPS may not be directly involved in inducing corrosion of metals, the presence of EPS in the biofilms helps in creating an ideal environment that supports corrosion processes (Ford and Mitchell 1990).

The adhering properties of EPS also play an important role in cell aggregation and floc formation. EPS produced by bacteria in granular sludge has been considered essential for the formation and maintenance of the microcolony (Tago and Aida 1977, MaCleod et al 1995). In sediments, bacterial EPS help in maintaining anaerobic conditions within the flocs. Such distribution of EPS also helps in spatial distribution of different micro-organisms within the flocs. For example, cell flocs in sediments have aerobes growing at the margins of flocs and anaerobes growing at the center. The presence of aerobes and greater concentration of EPS in the middle portion of cell flocs help maintain the anaerobic environment essential for the anaerobes (Murlidharan et al 1997).

Apart from adhesion, EPS in biofilms have also been implicated for motility in bacteria and diatoms. The role of EPS in motility has been recognised in some gliding bacteria (Decho 1990). Similarly, secretion of EPS by diatoms has been shown to aid motility (Medlin et al 1986, Pickett-Heaps et al 1991). In sediments, motile diatoms use EPS as a vehicle to migrate within the sediment layers in response to changing light and aeration conditions (Harper 1977, Moss 1977). Apart from aiding motility, the hydrated state of EPS (~99% water) provides protection to the cells against desiccation. Such properties enhance the survival of microorganisms in the inter-tidal mudflats that remain exposed during low tides (Decho 2000). The presence of water helps the EPS behave like a sorptive sponge that enables it to sequester nutrients, dissolved organic matter and dissolved metals (Decho 1990, Decho

and Lopez 1993). EPS in biofilms also contain localized enzymes that have been adsorbed from the surrounding environments (Lamed et al 1983, Costerton 1984, Decho 1990). The EPS-enzyme complex protects the enzymes from lysis but still retains its activity and accessibility to substrates. The biofilm EPS can also adsorb extracellular DNA released during cell lysis, thereby preventing the enzymatic degradation of DNA (Decho 1990). The transfer of plasmids between strains has been reported in *E. coli* biofilm (Lebaron et al 1997). The complex structure and chemistry of the EPS has been considered important in its ability to resist easy enzymatic degradation in the guts of animals (Plante 2000). EPS coated cells are frequently ingested by deposit feeders while feeding on sediment biofilms. However, such cells have been found to escape digestion, which are frequently excreted by the grazing community (Decho 1990, Gonzalez et al 1990, Jürgens and Güde 1994).

1.1f Applications of EPS

The unique properties of EPS have found varied applications in diverse fields. Moreover, its easy handling and eco-friendly production has made microbial EPS a lucrative product for industries. EPS like xanthan gum are widely used as food additives, stabilizer for beverages, reduction of crystallization in, as thinning agent and suspending agent (Sutherland 1998). Another bacterial EPS such as cellulose has found its utility in hospitals and acoustic diaphragms due to its greater water holding capacity, oxygen permissibility and tensile strength (Byrom 1991, Joris and Vandamme 1993). Polymers like β-D-glucans are used as anti-tumor agents. On the other hand, polymers like

heparin and hyaluronic acid are used in surgery, pharmaceutical products and cosmetics (Sutherland 1998). The adhesive properties of the EPS make it a good natural source of gelling agent, binder and glue (Sutherland 1998). EPS are also used in bioremediation of pollutants, in printers, oil recovery, coating of medicines and food items etc. (Sutherland 1998).

1.2 EPS in marine environment

1.2a Sources of EPS

Microorganisms like phytoplankton (diatoms) (Hellebust 1965, Myklestad 1977, Underhill 1981, Goto et al 1999), bacteria (Brophy and Carlson 1989, Goutx et al 1990, Heissenberger et al 1996), cyanobacteria (Singh et al 1999, Rougeaux et al 2001) and dinoflagellates (Guillard and Wangersky 1958, Alldredge et al 1998, Brachvogel et al 2001) release exopolysaccharides in the marine environments. Phytoplankton is considered to be the major source of organic matter in the euphotic waters of marine environments.

The fixing of CO_2 in presence of sunlight and inorganic nutrients like ammonia, nitrate and phosphates and its conversion to different organic biomolecules (both cellular and extracellular) and the subsequent utilization of the organic matter (respiration) is called primary production (PP) (Lalli and Parsons 1997). A major fraction of the primary production is released into the aquatic environments as extracellular polysaccharides (EPS) (Sondergaard et al 1985, Sell and Overbeck 1992). The production of EPS and its contribution

to the dissolved organic matter (DOM) has been reported for both free living (Myklestad 1977, Smestad et al 1981, Vaarum and Myklestad 1984, Myklestad 1995) and benthic forms (Bhosle et al 1995, Becker 1996, Buzelli et al 1997, Smith and Underwood 1998 & 2000, Penna et al 1999, Goto et al 2001) of phytoplankton. Putter (1909) hypothesized DOM as leachate of phytoplankton until Thomas (1971) gave experimental evidence that the production of EPS and its release as DOM was a normal physiological process in phytoplankton. The fraction of primary production released as extracellular organic carbon (EOC), however varies to a large extent and is influenced by method employed for estimation, species used and growth conditions (Eppley and Sloan 1965). Generally, the fraction of PP released as EOC ranges from <3% to more than 90% (Hellebust 1967, Wallen and Geen 1971, Raymont 1980, Gomes et al 1991, Sell and Overbeck 1992). The EOC produced by phytoplankton is principally made of polysaccharides (Wangersky and Guillard 1960, Ittekkot et al 1981, Hama and Yanagi 2001). Other organic compounds like free amino acids and dissolved proteins (Laane 1983, Striquer-Soares and Chevolot 1996) are also found in varying amounts. Moreover, Kirchman et al (2001) have reported that this extracellular polysaccharides released during blooms constituted up to ~50% of the semi-labile DOC, thereby forming a large fraction of the semi-labile DOC pool in oceanic waters.

The other source of EPS in marine environments is bacterioplankton population. These microorganisms range between 10^6 to 10^9 ml^{-1} in the marine waters (Cho 1991, Ramaiah et al 1998, Bode et al 2001), are

ubiquitously distributed (geographically and spatially) and form the largest living surface in the oceanic environment (Stoderegger and Herndl 1998). Unlike phytoplankton that grows only in the euphotic zone of the oceans (< 1% of the total ocean area), bacteria have been isolated from practically all depths and from extreme environments including hydrothermal vents (Cowen et al 1985, Winn and Karl 1987, Marteinsson et al 1995), deep sea sediments (Deming 1985, Hamamoto et al 1995) and cold seeps (Karl 1987). Moreover, under oligotrophic conditions the bacterial biomass may exceed the phytoplankton biomass even in euphotic zones (Herndl 1991, Fuhrman et al 1989, Cho and Azam 1990). Most bacteria produce capsules as long as they are metabolically active, which is then released into the surrounding waters as DOC (Brovy and Carlson 1989, Tranvik 1993, Heissenberger and Herndl 1994, Stoderegger and Herndl 1998, Heissenberger et al 1996). Bacteria utilize ~70% of phytoplankton EOC of which 45% is converted into EPS (Stoderegger and Herndl 1998). On the other hand, recent studies on EPS of marine bacteria have shown that these organic matter may not be easily degradable unlike phytoplankton EPS and behaved like humic substances (Brovy and Carlson 1989, Tranvik 1993). However unlike phytoplankton EPS, the significance of bacterial EPS in marine environments is not clearly understood and further studies are required to better understand the contribution of bacterial EPS to the DOC pools, its turnover rates and its role in marine food-web and biogeochemical processes.

1.2b Distribution of EPS in marine environments

In the marine environments, microbial EPS exists in various size classes- dissolved, colloidal and particulate forms. In the dissolved form, EPS forms a part of dissolved organic carbon pool ranging from a few kDa to more than 50,000 kDa molecular weight size (Decho 1990). In the form of colloids, EPS exists as particles in the size range of few nm to few µm in length (Cauwet 1978, Hedges 1987, Druffel et al 1992) and account for up to 40% of the organic carbon pool (Benner et al 1992, Ogawa and Ogura 1992, Wells and Goldberg 1992, Kepkay et al 1993). In the particulate form, EPS exists either as free discreet particles ranging from few µm to few hundred µm in length (Alldredge et al 1993, Mari and Kiørboe 1996, Mari and Burd 1998, Schuster and Herndl 1996) or in association with organic particles as in marine snow and other macroaggregates (Alldredge and Silver 1988, Cowen 1992, Herndl 1993, Decho and Herndl 1995, Unanue et al 1998). EPS in the dissolved form is directly estimated as dissolved carbohydrates. In contrast, EPS associated with cells in cultures have to be extracted using distilled water (Pfetzing et al 2000), saline (Underwood et al 1995), EDTA (Decho 1990) or by alkali treatment (Bhosle et al 1996).

1.2c Transparent exopolymer particles (TEP)

TEP are one type of EPS that are produced by both diatoms and bacteria during their growth (Alldredge et al 1993) (**Fig. 1.3**). These particles are found ubiquitously distributed in the marine environments and are produced from

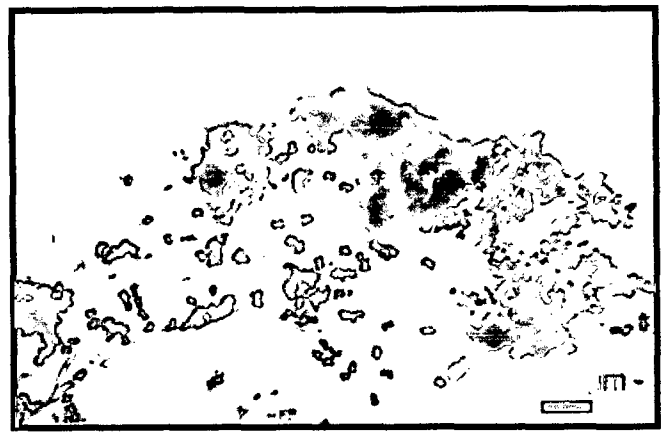

Fig. 1.3 Photograph showing the presence of Transparent exopolymer particles in natural sample. The particles stained blue with alcian blue (pH 2.5) and is associated with cells of *Phaeocystis* sp.

EPS released during the course of a phytoplankton bloom (Alldredge et al 1993, Passow and Alldredge 1994, Passow et al 1995, Schuster and Herndl 1996, Hong et al 1997, Krembs and Engel 2001, Berman and Vinner-Mozzinni 2001, Garcia et al 2002). Recent studies have shown that TEP can be generated from DOC derived from bacteria (Stoderegger and Herndl 1998) and phytoplankton (Passow 2000 & 2002) even in the absence of growing cells. Apart from phytoplankton and bacteria, TEP is also produced from other sources like mucus secreting bivalves (McKee et al 2000) and benthic macroalgae (Ramaiah et al 1999). TEP abundance ranges from 1 to 10^4 ml^{-1} in near coastal, shelf and open seas. The TEP size varies from < 2 µm to > 500 µm (Passow 2002).

Chemically, these particles are made of acid polysaccharides and are stained by polysaccharide specific anionic stains like alcian blue (pH 2.5) (Alldredge

et al 1993). The amount of TEP varies with source of the EPS (Stoderegger and Herndl 1998, Passow 2000 & 2002), age of the bloom (Kiørboe and Hansen 1993, Passow and Alldredge 1994, Hong et al 1997) and the presence of bacterial heterotrophs (Smith et al 1995) that may degrade the TEP. Due to its ability to coagulate, TEP is also considered important in flocculation of diatoms (Herndl 1993, Passow et al 1994, Dam and Drapeau 1995, Passow and Alldredge 1995, Grossart et al 1997, Engel and Schartau 1999, Brachvogel et al 2001) and their eventual sedimentation at the end of phytoplankton blooms (Crocker and Passow 1995, Logan et al 1995, Reibesell et al 1995, Passow et al 2001, Prieto et al 2001).

1.3 Significance of EPS in the marine environment

The distribution of EPS over a range of size class, its overall contribution to the total organic carbon pool and its unique properties makes it an integral component of biogeochemical processes, cycling of elements (C, N, S) and metals and the food web in the marine environments (**Fig. 1.4**). As evident from the figure, EPS released as DOC by phytoplankton and bacteria plays an important role in the carbon cycling within the water column. Dissolved EPS is involved in the production of TEP, which in turn helps aggregation of cells and formation of marine snow. On the other hand, the bacterial uptake of dissolved EPS released by phytoplankton alongwith nutrients convert the DOM to POM in the form of bacterial biomass. Moreover, the EPS released by bacteria and diatom also participate in the aggregation of live cells, detritus and cell debris, thereby forming additional POM (Biddanda 1985). The

colonization of these particles by particle-associated bacteria results in the degradation of POM, thereby releasing dissolved organic matter that adds to the ambient DOM concentrations and serves as additional organic carbon to free-living bacteria. Also, the POM produced by bacteria forms an important source of organic carbon for bacterivores like flagellates, ciliates and other forms of microzooplankton, which in turn is grazed upon by higher organisms. This simultaneous interconversion of POM to DOM during bacterial growth and the subsequent remineralization of POM by both bacteria and microzooplankton (especially flagellates) constitutes an important alternate pathway of organic carbon within the marine food-web called the microbial loop (Azam et al 1983). The dissolved EPS and TEP can bind and remove dissolved metals from the water column, thereby helping in recycling of metals. On the other hand, EPS associated with particulate matter including marine snow, large phytoplankton aggregates and its associated bacterial population may serve as a carbon source for grazers in the pelagic layer. Upon sedimentation of aggregates, EPS associated with these aggregates might serve as carbon source for benthic feeders before getting buried into the sediments. All these suggests that understanding the dynamics and properties of microbial EPS may help in better understand the mechanisms controlling the biogeochemical processes and food-web in the marine environments.

1.3a Role of EPS in aggregation

Ever since the discovery of macroaggregates like marine snow (Suzuki and Kato 1953) in the pelagic waters, numerous studies have been carried out to explain the mechanisms of macroaggregate production (McCave 1984, O'Melia 1987, Jackson 1990, Eisma 1993, Kepkay 1994, Zhou et al 1998) and the essentials of aggregation (O'Melia 1987, Eisma 1993, Jackson et al 1994, Stumm et al 1994). Aggregation in the natural environment involves both biotic and abiotic processes. In the biotic process, live cells of phytoplankton and bacteria are the presence of living cells is considered essential for the production of macroaggregates. For example, Biddanda (1985) reported the formation of macroaggregates, which were essentially clumps of bacterial cells glued together by EPS during the microbial degradation of dissolved organic matter. Similarly, aggregate associated bacteria utilize and convert DOM into fibrillar EPS that aids in aggregation (Paerl 1974, Leppard et al 1979, Heissenberger et al 1996). Production of TEP by living microbial cells is also considered an important mechanism of aggregation during phytoplankton blooms (Alldredge et al 1993, Passow and Alldredge 1995).

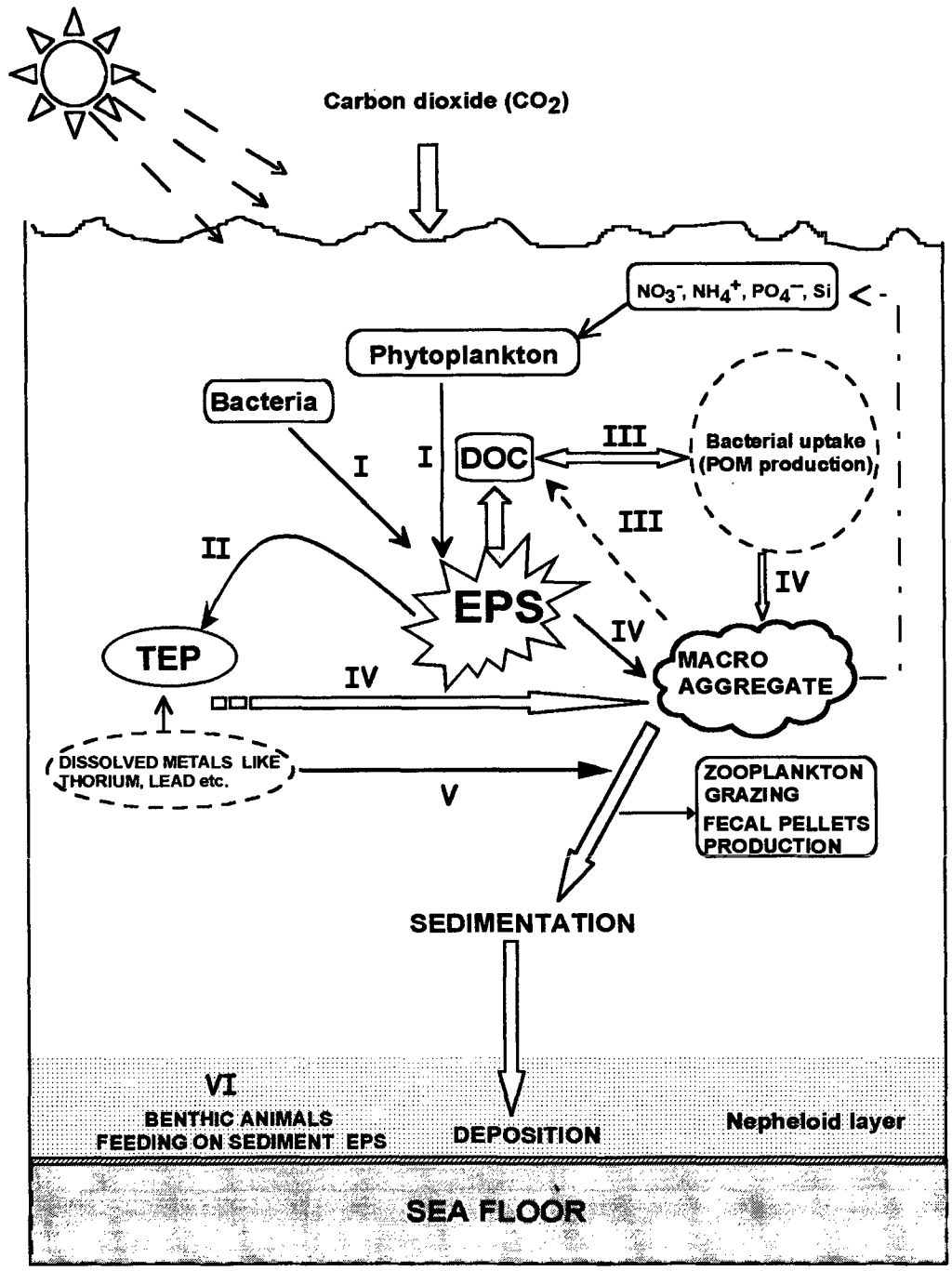

Fig. 1.4 Schematic representation of the various roles of EPS in the marine environment. The numbering indicates the different processes involving EPS.
I- Production of EPS by bacteria and phytoplankton; II- Production of TEP from EPS; III- Microbial loop; IV- Formation of particles; V- Chelation of dissolved metals and VI- EPS as a carbon source for benthic community.

In contrast to the biotic form of aggregation, EPS in its dissolved form can get converted to form discreet particles in abiotic conditions. Under abiotic aggregation, particles might be produced from dissolved EPS either due to shear (Jackson 1990, Reibesell 1991), surface adsorption of particles (Gibbs 1983, Eisma 1993, Johnson et al 1994), surface coagulation of EPS (Mopper et al 1995, Zhou et al 1998, Mari 1999) and self-coagulation of EPS (Kepkay 1994, Chin et al 1998, Passow 2000). Moreover, free EPS may act as a mesh that entraps particles (Logan and Hunt 1987, Logan and Alldredge 1989, Stolzenbach 1993). EPS may get adsorbed to the surface of colliding particles and allows them to stick together and form macroaggregates. This process is common in the marine environments and is influenced by particle density, collision frequency and the binding properties of the EPS (McCave 1984, Kiørboe et al 1994, Waite et al 1997, Engel 2000).

1.3b EPS in particle sedimentation and carbon flux

The sinking of particulate matter from the surface to the deeper waters is called as sedimentation. This process generally coincides with increase in concentration of EPS at the termination of phytoplankton blooms (Passow and Alldredge 1995). In the natural environments, microbial cells are buoyant in nature and do not sink by themselves. However in presence of extracellular compounds like EPS, they tend to coagulate along with other particulate matter to form macroaggregates as described earlier, attain a critical mass and start sinking into the deeper waters. Sedimentation is considered essential for the survival of phytoplankton cells in the marine environments.

For example, sedimentation of phytoplankton cells is essential to escape predation, nutrient depleted conditions and transportation to favorable growth conditions (Smetacek 1984). Moreover, spores of diatoms also aggregate and sink into deeper waters, serving as seed for future blooms (Smetacek 1984). The fate of sedimenting particles is also controlled by EPS. In the open ocean, it is possible that only 10% of the photosynthetic carbon escapes the euphotic layer as sinking particles (Fowler and Knauer 1986), of which only 1% reaches the deep-sea sediments (Martin et al 1987, Wefer 1989). One reason for such low organic carbon flux may be the tightly coupled response of grazers and bacterial community to the EPS produced by phytoplankton. For example, the sinking aggregates coated with EPS serve as carbon source for grazers like zooplankton in the water column (Gotschalk and Alldredge 1989, Lampitt et al 1993). Similarly, particle associated bacteria might utilize the EPS coatings using an array of extracellular enzymes and release it in the dissolved form (Azam 1998). Such a response would lead to disintegration of the sinking macroaggregates and most of the organic matter might get remineralized in the euphotic zone.

On the contrary, formation of EPS coated macroaggregates and its sedimentation indicates an uncoupling between the grazers and phytoplankton population. Various factors including EPS chemistry, resistance to bacterial breakdown of EPS and low grazing of aggregates are attributed to the formation and sedimentation of macroaggregates in marine waters. For example, EPS produced by *Phaeocystis* might resist bacterial mineralisation and lead to the formation of large aggregates (Eberlein et al 1985, Billen and

Fontigny 1987). Moreover, depletion of essential nutrients like nitrogen and phosphorus (Pengerud et al 1987) and adsorption of colloids (Johnson and Kepkay 1992) can also limit the bacterial solubilization of phytoplankton EPS. Similarly, presence of EPS may not always support the grazing activity of higher organisms. Studies have shown that TEP produced during blooms of *Thallasiosera* caused aggregation as grazers like copepods did not prefer to consume TEP-coated particles (Preito et al 2001). All these indicate that quality of EPS and its fate is directly linked to the quality of particles reaching the sediments, thereby influencing the benthic community structure and biomass.

1.3c EPS in microbial loop

Since a major fraction of the photosynthetic carbon (> 90%) might be released as soluble EPS (Sell and Overbeck 1992), much of the photosynthetic carbon remains unavailable to higher organisms and escapes the classical food-chain. However, the soluble form of EPS released by the phytoplankton serves as an organic carbon source and is degraded and converted to particulate form by marine bacteria (Decho and Herndl 1995, Azam 1998). On an average about 50% of the photosynthetic carbon is channelised through bacteria within the microbial loop (Fuhrman 1992), although the amount may vary considerably. The decomposition of aggregates, its dissolution and mineralisation completes the microbial loop (Azam et al 1991, Azam 1998) (**Fig. 1.4**). The bacterial conversion of the dissolved EPS to particulate organic matter (POM) is critical in meeting the carbon demand of secondary

consumers. The POM so formed might become a source of carbon for grazers like ciliates, flagellates etc., thus channelizing the organic carbon into the classical food-chain. The significance of microbial loop is not restricted to marine food-web but also in controlling the atmospheric carbon dioxide levels. Globally, the amount of carbon dioxide assimilated into the microbial loop after photosynthesis is reported to be equivalent to 5 times the anthropogenic carbon dioxide emission (Williamson and Holligan 1990). Thus, the quality and the quantity of EPS released by the phytoplankton is important in regulating the efficiency of marine microbial loop.

1.3d Removal of metal ions and its cycling

Microbial EPS are known for their ability to bind a wide range of metals including heavy metals like Pb, Co, Sr, Cr, Th and Cd (Dugan and Pickrum 1972, Corpe 1975, Brown and Lester 1982). Microbial EPS isolated from biofilms have been most often used to study the binding of metals (Harvey 1981, Mittleman and Geesey 1985, Ford et al 1987). Both laboratory and field studies have shown that factors including pH, EPS chemistry and structural orientation of the polymer influence the metal binding efficiency of the EPS in natural environment. In the marine environments, bacterial capsules are known to bind metals in both sediments (Nealson 1983) and in the water column (Cowen and Silver 1984, Cowen and Bruland 1985). Binding of metal ions by microbial EPS may have several implications. For example, dissolved ions bound by EPS are considered essential in the vertical transport of these ions. EPS in aggregates have been reported to contain metal precipitates that

accumulated with depth (Decho 1990). Once the metal ions reach the sediments, it may undergo microbial transformations and leached back into the overlaying waters. On the other hand, particulate EPS like TEP can bind more than 90% of the dissolved thorium ions and thereby alter the rate of removal of thorium from pelagic waters (Quigley et al 2002). Since thorium is used as a proxy to estimate the flux of organic carbon in the open ocean, such binding of thorium to TEP can lead to discrepancy in the estimates of carbon flux (Passow 2002).

1.4 Negative impact of EPS

Production of large aggregates in coastal waters can also be a nuisance factor since it may lead to organic loading in these waters. Mucilaginous polysaccharides of certain phytoplankton form large aggregates that sink to bottom sediments (Herndl and Peduzzi 1988, Stachowitsch et al 1990). Such accumulation of aggregates increases the oxygen demand, causing anoxic conditions in the bottom waters. These anoxic conditions cause large-scale mortality of benthic fauna in these areas (Haumann 1989, Rogers and Lockwood 1991). Similarly, near bottom anoxic waters would lead to anaerobic degradation of organic matter, leading to increased production of greenhouse gases like H_2S and N_2O (Naqvi et al 2000). Since EPS are good adsorbents of metals and other pollutants, the accumulation of EPS in coastal waters can lead to chelation of these pollutants. The grazing of these aggregates can transfer the bound metals to higher trophic levels in the food-chain. Such process is involved in the accumulation of pollutants in the

tissues of higher organisms (Decho 1990). Microbial EPS also play an important role in the fouling and corrosion of economically important submarine structures, thereby causing damage to property and economic losses (Maki 1999).

1.5 Aims of the thesis

Although studies have been conducted to address the importance of microbial EPS in the marine environments, there is no information on the dynamics of EPS and its relationship with heterotrophic activity and production in natural waters, especially in shallow coastal waters. Moreover, there is not much information on the role of bacterial EPS in marine biogeochemical processes and food-web. In this research, an attempt has been made to understand the importance of microbial EPS in various marine processes. The first objective was to understand the dynamics of EPS and its relation to bacterioplankton abundance and productivity, abundance of TEP and extracellular glucosidase activity at a shallow water coastal station at Dona Paula Bay, west coast of India. The second objective was to assess the production of EPS by a marine bacterium and evaluate the chemical characteristics of the EPS produced by the marine bacterium. This chemically characterised EPS was then used to define its role, if any, in the production of macroaggregates. Further, the bacterial EPS was also assessed to define its role as an organic carbon source to heterotrophs. In this regard, laboratory experiments were conducted using benthic polychaete *Nereis (Hediste) diversicolor* (O F Müller 1876) and

natural marine bacterial population. Moreover, the role of EPS in binding of metals like Pb and Cu was also assessed using *Marinobacter* EPS.

The results presented in this study indicate that EPS in the marine waters varied seasonally and supported bacterial production. However, phytoplankton was not the only source of EPS in the shallow water coastal station at Dona Paula Bay, west coast of India. EPS isolated from *Marinobacter* sp. and *S. costatum* were able to form macroaggregates (max. size of 22.7 and 34.5 mm^2, respectively) within 30 h of incubation in rolling tanks. *Marinobacter* EPS was ingested and assimilated in significant amounts by benthic polychaete *Nereis (Hediste) diversicolor*. Moreover, natural bacterioplankton was able to rapidly breakdown and assimilate *Marinobacter* EPS within the first 24 h of incubation. Furthermore, the bacterial EPS was found to efficiently bind more of free copper than lead ions.

CHAPTER 2A

Dynamics of particle-associated exopolysaccharides (EPS) in the Dona Paula Bay

Chapter 2a

2A.1 Introduction

Exopolysaccharides (EPS) are high molecular weight compounds and are sticky in nature (Sieburth et al 1976, Alldredge and Silver 1988, Alldredge et al 1993, Passow et al 1994). In the aquatic environments, phytoplankton blooms are one of the major sources of EPS with as much as 80% of fixed carbon being released into the surrounding waters (Myklestad and Haug 1972, Myklestad 1977, Myklestad et al. 1989). EPS is also derived from other sources like bacterial growth (25% of respired carbon), sloppy zooplankton feeding and cell-lysis etc. (Decho 1990, Kaltenböck and Herndl 1992, Stoderegger and Herndl 1998, Jacobsen 2000, Dilling and Alldredge 2000). In coastal waters, benthic macroalgae, microphytobenthos and land-derived organic matter may also act as additional sources of EPS.

The production and chemical characteristics of EPS are controlled by nutrient dynamics, microbial physiology, phytoplankton species, age of phytoplankton bloom etc. Similarly, EPS produced by microorganisms may exist either as tight capsules or as loosely attached slimes (Decho 1990, Bhosle et al 1995, Underwood et al 1995) that generally differ in their physico-chemical characteristics (Decho 1990, Hoagland et al 1993). For example, capsular EPS are less degradable and may persist for a longer period in the water column than slime EPS (Stoderegger and Herndl 1998). On the other hand, slimes play an important role in flocculation and mass sedimentation of

phytoplankton blooms (Kiørboe and Hansen 1993). Such differences in their chemistry and properties may lead to varying behavior of EPS in time and space.

EPS may directly or indirectly contribute to particulate (Passow et al 1994, Baldi et al 1997), colloidal (Sharp 1973, Hung et al 2001) or dissolved organic matter (Burney et al. 1979, Benner et al 1992, Borch and Kirchmann 1997, Myklestad et al 1997). Apart from several important functions including cell attachment, biofilm development, sequestering nutrients etc. (Decho 1990, Wingender et al 1999), EPS is also involved in stabilization of sediments (Decho 2000), organic carbon transformations through the microbial loop (Cho and Azam 1988, Decho and Herndl 1995) and metal cycling (Passow 2002) in the natural environments. EPS may also serve as an alternate carbon source for marine bacterioplankton (Fajon et al 1999, Goto et al 1999).

These polymers may coagulate spontaneously to form discreet exopolymeric particles called transparent exopolymeric particles (TEP) by biotic and/or abiotic processes (Alldredge et al 1993, Mopper et al 1995, Chin et al 1998, Zhou et al 1998). TEP are generally abundant during the decline of a bloom (Alldredge et al 1993, Passow and Alldredge 1994, Mari and Kiørboe 1996). Due to their global distribution, EPS may be considered the second largest organic carbon pool after dissolved organic carbon and contributes directly to the cycling of organic carbon (Benner et al 1992, Kepkay 1994, Amon and Benner 1994, Mari 1999).

Various methods have been adopted to isolate EPS from natural samples including high-speed centrifugation (Decho 1990), alkali, EDTA and 1.5M NaCl solution (Bhosle et al 1995, Underwood et al 1995). Of all the method, EDTA has been widely used to extract/isolate tightly bound EPS in both cultures and natural samples (Decho 1990, Underwood et al 1995). On the other hand, 1.5M NaCl treatment was used to isolate loosely bound EPS from diatoms without damaging the fragile cell-wall (Bhosle et al, 1995). Since the extraction of EPS depends upon the chemistry of the EPS and the characteristics of the treatment, both extraction methods were employed. Therefore, it is possible that extraction of natural samples using these two different techniques might yield EPS having different chemical characteristics.

Despite the numerous functions ascribed to EPS and TEP in aquatic environment, there is little information on their dynamics in near-shore, especially tropical waters. Most of the reports on the dynamics of EPS and/or TEP in near shore waters are either from temperate or polar waters (Kiørboe et al 1994, Schuster and Herndl 1995, Riebesell et al 1995, Hong et al 1997, Moreira and Martin 1998). In view of the above, the work presented in this chapter was planned to 1) assess the temporal variations of bacterial abundance, EPS (NaCl-soluble EPS and EDTA-soluble EPS) and TEP concentrations in the suspended particulate matter and sinking macroaggregates, 2) evaluate the influence of these polysaccharides on bacterioplankton abundance and 3) assess the contribution of these polysaccharides to the POC. These parameters along with hydrography and

nutrients were monitored over a period of 26 months at a shallow water station in the Dona Paula Bay.

2A.2 Materials and Methods

2A.2.1 Sampling site

The sampling site was the Dona Paula jetty (15°27'N; 73°48'E), which had an average depth of 3m (**Fig. 2A.1**). The area experiences three seasons viz.: (1) southwest monsoon (June-September) when the area is inundated with riverine water and land runoff due to heavy precipitation. This brings about major changes in temperature, salinity, suspended load, dissolved oxygen, nutrients and organic matter composition. The water column near the study

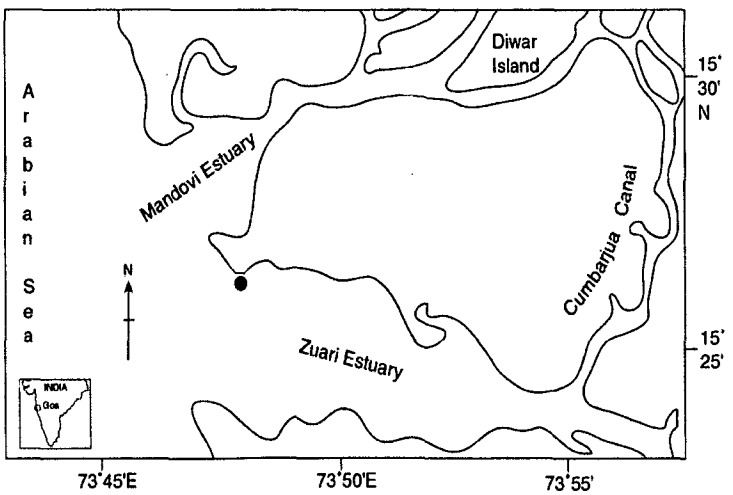

Fig. 2.1 The location map of the study site.

site experiences stratification due to fresh water influx during monsoon; (2) a

post monsoon (October-January) season which is a recovery period from freshwater domination with increase in salinity but little changes in surface water temperature and (3) a premonsoon season (February-May) when the water becomes typically marine (Qasim and SenGupta 1981, Gauns 2000). Earlier studies at this study site showed that the surface water temperature varied from 25°C to 33°C, whereas the surface salinity ranged from a low of 10 psu (during monsoon) to a high of 35 psu (pre-monsoon). Moreover, the study site is close to the mouth of a tidal estuary and hence experiences tidal currents ranging from 1 cm s^{-1} in the post-monsoon to 25 cm s^{-1} during late pre-monsoon months (Jayakumar, personal communication). The tidal range in the study area varied from 0.25 m (neap tide) to 2 m (spring tide) (Chandramohan et al 1998). Since the sampling site was close to the shore, it experiences heavy wave action during monsoon. The resuspension of bottom sediment is high during low tides and monsoon months. The mean significant wave height reported in these waters was 0.93 ± 0.18 m and the frequency of maximum height waves was 10 s (Vethamony and Kumar 1997). Nitrate levels at the site vary from 1 µM in the pre-monsoon to 14 µM during monsoon (Devassy and Goes 1988). The site receives terrestrial organic inputs from the Zuari and Mandovi estuaries, which are fringed with thick mangrove coverage (Wafar et al 1997). The phytoplankton or micro-algal population is one of the major sources of organic carbon in the Dona Paula Bay with periodic blooms of diatoms like *Skeletonema*, *Nitzschia*, *Navicula*, *Coscinodiscus* and *Chaetoceros* during the months of September-October, December-January and April (Devassy and Goes 1988, Gauns 2000). Beds of macroalgae like *Sargassum* appear near the study site during the post-

monsoon months. Moreover, the study site is a tourist spot and therefore experiences intense human activities.

Sampling

Surface seawater samples were collected using an acid clean plastic carboy during high tide at fortnightly intervals during 1998-99 and thereafter once a month in 1999-2000 for the analyses of various parameters. Macroaggregate samples were collected with a slight modification of the method of Grossart et al (1998). Briefly, a 5 l Niskin water sampler whose lower lid was kept closed and suspended at 1m below the surface. After 30 mins, the upper lid of the Niskin sampler was closed using a messenger and the sampler was retrieved. The sampler was brought to the laboratory and kept in an upright position for one hour to allow the macroaggregates to settle. The supernatant was then siphoned off and the content of the sampler was adjusted to a known volume. Both surface seawater and macroaggregate samples were prefiltered through 20 µm nylon mesh to remove large swimmers before filtering for different parameters.

Aliquots of seawater or macroaggregates were filtered onto preweighed ashed GF/F filter papers for various particulate parameters like dry weight, chlorophyll *a* (Chl*a*), particulate organic carbon (POC) and EPS (NaCl-soluble EPS and EDTA-soluble EPS). All the particulate samples except Chl *a* samples were oven dried at <45°C.

TEP samples were collected as described by Alldredge et al (1993) except that 0.22µm pore-sized Nuclepore polycarbonate membrane filters were used instead of 0.45µm pore-sized filters. Twenty five to fifty ml of seawater or macroaggregate samples for quantitative estimation of TEP and 1 or 2 ml for microscopic enumeration and size estimation of TEP were filtered onto 0.22µm Nuclepore polycarbonate membrane filters using <10 mbar pressure. The sampling was carried out in duplicates.

Sub-samples of seawater and macroaggregates were fixed with formalin (4% final concentration) for the enumeration of total bacteria (Parsons et al 1984).

2A.2.2 Wind speed, hydrography and nutrients

Surface wind speed data were obtained from the local meteorological station (Meteorological Station, Altinho-Panjim). Sea surface temperature was recorded *in situ* using a thermometer. Seawater samples were brought to the laboratory within half an hour of collection and analyzed for salinity, dissolved oxygen and nutrients (nitrate & phosphate). Salinity was estimated following the silver nitrate titration method (Stickland and Parsons 1975). Samples for dissolved oxygen were fixed and analyzed following the Winkler's method (Parsons et al 1984). Dissolved nitrate and phosphate were estimated spectrophotometrically (Parsons et al 1984). All samples were analyzed in duplicates.

2A.2.3 Suspended particulate matter and Macroaggregates

2A.2.3a Dry weight

Filter papers with suspended particulate matter (suspended load) or macroaggregate (total macroaggregates) were washed with distilled water, oven dried at ~45°C and weighed on a microbalance (Model Mettler Toledo-AT 20) to estimate the dry weight.

2A.2.3b Chlorophyll a (Chl a)

The Chl *a* samples were extracted overnight in 90% acetone. After extraction, the samples were centrifuged and the supernatant was measured for its fluorescence at 430nm excitation and 670nm emission wavelengths using a fluorescence spectrophotometer (Hitachi model F-2000). The method was calibrated using standard chlorophyll *a*.

2A.2.3c Particulate organic carbon (POC)

The POC samples were analyzed following the dichromate oxidation method (Parsons et al 1984). Briefly, the filter paper containing particulate matter was taken in acid-cleaned test-tube and treated with 1 ml phosphoric acid. The samples were heated in a boiling water bath for 30 min. Thereafter, sulphuric acid-dichromate mixture was added and the tubes were further heated in

boiling water bath for 60 min. Samples were then diluted to a known volume using distilled water and cooled. Aliquots of the samples were centrifuged and the supernatant was measured at 440 nm. Plain filters were treated in the same fashion and used as blanks. Glucose was used as standard for the calibration of method.

2A.2.3d Exopolysaccharides (EPS)

Suspended particulate matter or macroaggregate samples were treated with 1.5M NaCl or 10mM EDTA so as to extract NaCl-soluble EPS or EDTA-soluble EPS, respectively (Bhosle et al 1995, Underwood et al 1995). Samples were extracted for 15 minutes using vortex mixer and then centrifuged at 3000 rpm (2062 g) for 10 mins. To 1 ml of the supernatant, 1 ml of cold 5 % phenol solution followed by 5 ml concentrated sulphuric acid were added. Samples were cooled and measured spectrophotometrically at 490 nm (Dubois et al 1956). Plain filters were treated as above and used as blanks. Glucose was used as a standard for calibration. Results are expressed as glucose equivalent.

2A.2.3e Bacterial abundance

Total bacterial abundance was estimated following the acridine orange staining method (Parsons et al 1984). Briefly, 100-200 µl of the acridine orange stain (0.1%) was added to duplicate sterile tubes containing 1-2ml of the formalin fixed samples and incubated in dark for 2 min. The samples were

then filtered onto 0.22µm black Nuclepore polycarbonate membrane filters. The filters were then placed on a drop of fluorescence free oil, another drop of oil placed onto it and covered with a cover slip. The slide was then viewed under oil immersion lens using a blue filter with a UV lamp as a source of light. About 10-12 fields were counted per filter and the average field count was used to calculate the abundance of bacteria as follows:

$$\text{Bacterial abundance/ml: Mean counts} \times \frac{\text{filter area}}{\text{field area}} \times \frac{1}{\text{ml filtered}}$$

2A.2.3f TEP- chemical quantification

In order to quantify TEP, filters containing the particulate matter samples were stained with prefiltered (0.22 µm) alcian blue (0.02% in 0.06% glacial acetic acid; pH=2.5) and filtered dry under low vacuum (Passow and Alldredge 1995b). Blanks were prepared by staining empty filters with alcian blue.
Both TEP and blanks were treated with 6 ml of 80% sulfuric acid for 2h with intermittent shaking. The samples were then centrifuged and the supernatant was measured spectrophotometrically at 787 nm (Passow and Alldredge 1995b). The TEP concentration was estimated as follows:

$$TEP = (E_{787} - B_{787}) \times F_x V_f^{-1}$$

where E_{787} is sample extinction, B_{787} is blank extinction, F_x is the factor of calibration and V_f is the volume filtered in litres. The factor F_x was calculated from the equation

$F_x = M[(E_{787} - B_{787})V_{st}^{-1}]^{-1}$;

where M is the weight of the standard (μg l^{-1} Xanthan gum) and V_{st} is the volume of the standard filtered in liters. Xanthan gum was used as a standard for calibration. The coefficient of variance for the entire procedure was 35%. The TEP concentrations are expressed as Xanthan gum equivalent.

2A.2.4 Statistical Analyses

In order to assess the correlation between various parameters, correlation matrices were obtained for both suspended particulate matter and macroaggregates. As the parameters showed strong seasonal variations and behaved differently in the two years, the correlation matrix was calculated for each year separately. The statistical analyses were carried out using STATISTICA 5.0.

2A.3 Results

2A.3.1 Surface Winds

During the sampling period, the wind speed varied from a high of 4.8 m s^{-1} (July 2000) to a low of 1.9 m s^{-1} (October 1999) (**Fig. 2A.2a**) with an average wind speed of 2.5 ± 0.77 m s^{-1}. Seasonally, the wind speed was higher (> 3 m s^{-1}) during south-west monsoon (June-September), reduced with the onset of post monsoon (October- January) season and did not fluctuate much during rest of the sampling period.

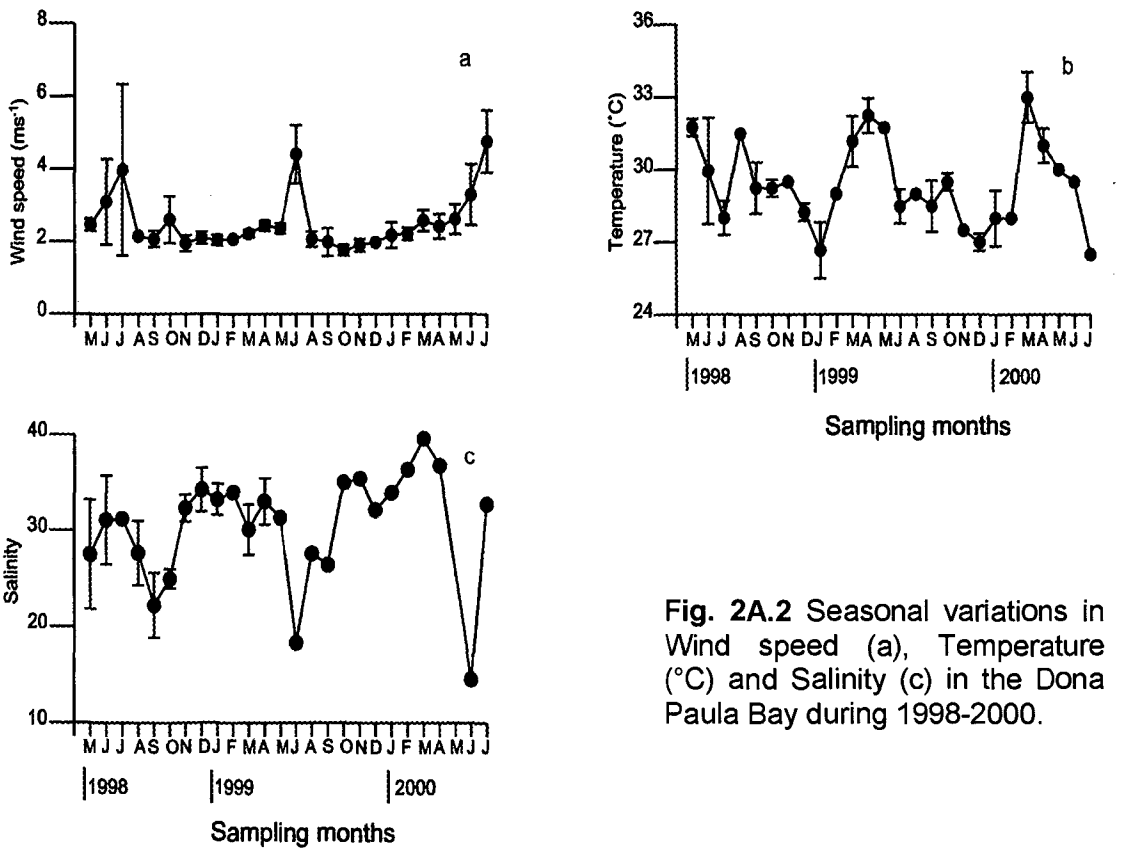

Fig. 2A.2 Seasonal variations in Wind speed (a), Temperature (°C) and Salinity (c) in the Dona Paula Bay during 1998-2000.

2A.3.2 Hydrography

The surface water temperature at the study site showed large-scale variations and ranged from 26.5°C (July 2000) to 33°C (March 2000) (**Fig. 2A.2b**). The surface water temperature was lowest in the post-monsoon (October-January) (26°C to 28°C), moderate in monsoon (June-September) (28 to 30°C) and higher in the pre-monsoon (February-May) (31°C to 33°C) during the 26-month study period. The surface salinity for the entire study period varied from 14.7 (June 2000) to 39.7 (March 2000) (**Fig. 2A.2c)**.

Over the period of sampling, the average dissolved oxygen concentration for the study period was 104.9 ± 11.5 µM. The seasonal variation in dissolved oxygen was conspicuous during the sampling period. The oxygen concentrations showed small variations between May '98 to March '99 and varied from 94 ± 28.1 µM (August '98) to120.6 ± 5.8 µM (March 1999) (**Fig. 2A.3a**). Subsequently, the oxygen values declined continuously until November '99 (78.8 ± 2.3 µM) except for a high in July '99 followed by a sharp increase in December 99 (123 µM). Thereafter the oxygen values did not vary much for the rest of the sampling period.

2A.3.3 Nutrients

The surface water nitrate concentrations displayed distinct seasonal rhythm and were influenced by the southwest monsoon (June-September). The nitrate concentration for the study period ranged from 0.03 µM (September '99) to 14.1 µM (July '99). The nitrate concentrations were higher in the monsoon (June-September) (5.7 ± 2.2 µM) and declined in the post-monsoon (October-January) season (2.5 ± 0.4 µM) (**Fig. 2A.3b**). Similarly, secondary maximal concentrations were observed in December '98 and March 2000. Unlike the nitrate, the phosphate concentrations in the surface waters did not show any specific seasonal trend (**Fig. 2A.3c**). On the whole, the phosphate concentrations varied 0.09 ± 0.03 µM (November '99) to 2.7 ± 2.9 µM (April '99).

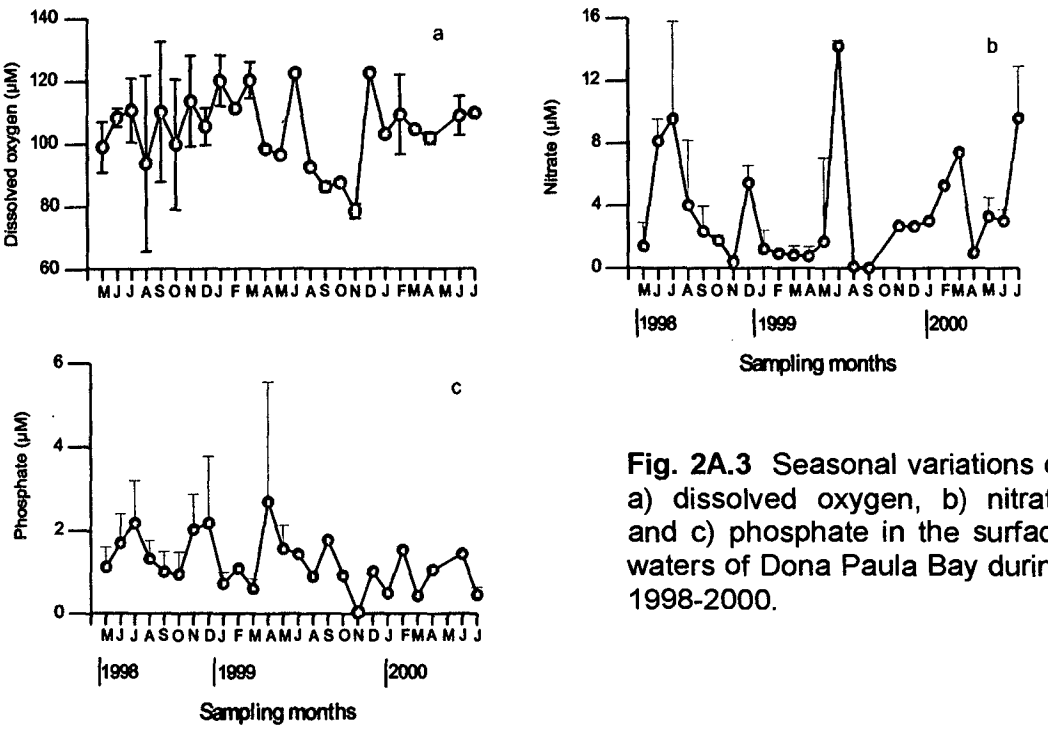

Fig. 2A.3 Seasonal variations of a) dissolved oxygen, b) nitrate and c) phosphate in the surface waters of Dona Paula Bay during 1998-2000.

2A.3.4 Dry weight

The dry weight of suspended particulate matter (suspended load) in the surface waters ranged from 3.4 (September '99) to 44.9 mg l^{-1} (June '98) (**Fig. 2A.4a**). The suspended load was higher during monsoon (June-September) (21.3 mg l^{-1}) and lower in post-monsoon (October-January) (11.4 mg l^{-1}) followed by a small increase in pre-monsoon (February-May) (14.3 mg l^{-1}) (**Table 2A.3**).

The macroaggregates dry weight ranged from 2.9 (February '99) to 185.5 mg l^{-1} (July '99) during the 26-month study and followed a seasonal pattern similar to that of suspended load (**Fig. 2A.4b**). The macroaggregate concentrations were higher in the monsoon (June-September) (45.7 ± 54.3 mg l^{-1}) and lower in post-monsoon (October-January) (20.7 ± 12.3 mg l^{-1}) (**Table 2A.4**).

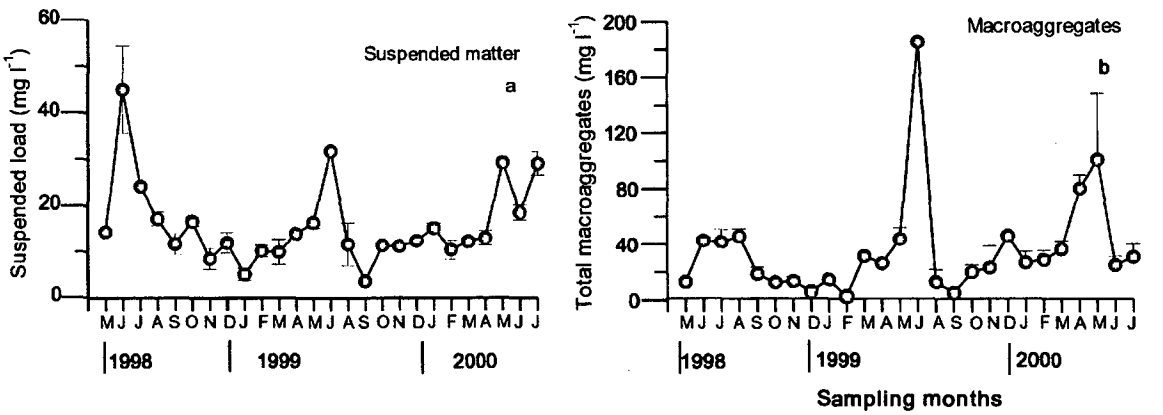

Fig. 2A.4 Seasonal variations in the concentrations of a) Suspended load and b) Total macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

Table 2A.1: Mean, SD, maximum and minimum range of various particulate parameters in the suspended matter during the period 1998-2000 in the Dona Paula Bay.

Parameters		Mean	SD	Max	Minimum
Suspended load	mg l^{-1}	15.9	9.2	45.0	3.4
Chl *a*	µg l^{-1}	4.8	2.7	12.3	1.2
POC	µg l^{-1}	700.5	256.1	1311.2	270.1
NaCl-soluble EPS	µg l^{-1}	24.5	20.3	101.6	4.5
EDTA-soluble EPS	µg l^{-1}	64.4	41.0	210	26.2
TEP	µg l^{-1}	55.6	35.6	149.1	1.3
TBA	x 10^6 cells/ml	2.4	2.0	8.2	0.5
Phyto-C (%POC)		32.2	20.4	71.6	5.5
Bac-C (% POC)		4.7	4.8	20.8	0.5
NaCl-soluble EPS- C (% POC)		1.6	1.2	5.4	0.4
EDTA-soluble EPS- C (% POC)		4.0	2.0	11.2	1.4
TEP-C (% POC)		6.9	5.9	22.8	0.1

Chl *a*: Chlorophyll *a*; POC: Particulate organic carbon; TEP: Transparent exopolymer particles; TBA: Total Bacterial Abundance; Phyto-C: Phytoplankton carbon; Bac-C: Bacterial carbon.

Table 2A.2: Mean, SD, maximum and minimum range of various particulate parameters in the macroaggregates during the period 1998-2000 in the Dona Paula Bay.

Parameters		Mean	SD	Max	Minimum
Total macroaggregates	mg l^{-1}	36.3	37.6	185.5	2.9
Chl *a*	µg l^{-1}	2.7	1.7	6.2	0.4
POC	µg l^{-1}	1116.1	960.3	3325.5	156.6
NaCl-soluble EPS	µg l^{-1}	24.0	21.7	84.5	0.9
EDTA-soluble EPS	µg l^{-1}	25.3	26.7	79.2	0.1
TEP	µg l^{-1}	17.8	17.5	72.3	0.4
TBA	x 10^6 cells/ml	2.6	2.1	9.5	0.5
Phyto-C (%POC)		19.3	18.1	62.7	0.4
Bac-C (% POC)		4.6	4.8	19.9	0.1
NaCl-soluble EPS-C (% POC)		1.7	1.7	6.4	0.1
EDTA-soluble EPS-C (%POC)		2.7	2.3	7.9	0.3
TEP-C (% POC)		2.0	2.0	4.9	0.02

Chl *a*: Chlorophyll *a*; POC: Particulate organic carbon; TEP: Transparent exopolymer particles; TBA: Total Bacterial Abundance; Phyto-C: Phytoplankton carbon; Bac-C: Bacterial carbon.

Table 2A.3 : Average concentration of the various parameters in the suspended particulate matter in different seasons.

Parameters	Monsoon	Post-monsoon	Pre-monsoon
Suspended load[1]	21.3 ± 12.6	11.4 ± 3.6	14.3 ± 6.4
POC[2]	760.1 ± 264.3	606.4 ± 182.2	708 ± 330
Chl *a*[2]	3.8 ± 2.5	5.6 ± 3.1	5.3 ± 2.5
NaCl-soluble EPS[2]	17.6 ± 6.9	33.8 ± 30.5	19.1 ± 12.5
EDTA-soluble EPS[2]	69.6 ± 24.3	66 ± 59.5	59 ± 41
TEP[3]	57.7 ± 44.6	50.1 ± 22.8	58.6 ± 38.6
TBA[4]	1.7 ± 1.4	4 ± 2.4	1.5 ± 1.1
%Phyto-C	24.9 ± 22.2	36.8 ± 15.5	38.7 ± 22.5
%NaCl-EPS-C	1.1 ± 0.7	2.2 ± 1.5	1.6 ± 1.4
%EDTA-EPS-C	4.0 ± 1.0	4.4 ± 2.9	3.8 ± 1.9
%Bac-C	2.9 ± 3.2	7.8 ± 5.9	3.4 ± 3.7
%TEP-C	6.8 ± 6.5	6.7 ± 4.4	7.1 ± 7.3

1: mg l^{-1}; 2: µg l^{-1}; 3: mg xanthan gum eq. l^{-1}; 4: x 10^6 ml^{-1}.

Table 2A.4 : Average concentration of the various parameters in the macroaggregate samples in different seasons.

Parameters	Monsoon	Post-monsoon	Pre-monsoon
Total macroaggregates[1]	45.7 ± 54.3	20.7 ± 12.3	40.7 ± 31.3
POC[2]	1491 ± 1118	439.2 ± 328.3	1122 ± 942
Chl *a*[2]	2.8 ± 1.9	3.0 ± 1.4	2.4 ± 1.8
NaCl-soluble EPS[2]	29.8 ± 26.8	25.5 ± 26.8	17.3 ± 9.9
EDTA-soluble EPS[2]	25.7 ± 26	22.8 ± 31.1	27.3 ± 29.6
TEP[3]	13.5 ± 19.1	13.1 ± 5.2	27.2 ± 21.2
TBA[4]	2.3 ± 2.9	3.8 ± 3.3	1.8 ± 1.5
%Phyto-C	23.6 ± 26	19.8 ± 8.2	12.1 ± 10.6
%NaCl-EPS-C	1.4 ± 1.2	2.7 ± 2.5	1.8 ± 0.9
%EDTA-EPS-C	3.2 ± 2.3	2.9 ± 2.8	1.8 ± 1.7
%Bac-C	3.8 ± 2.9	6.4 ± 6.8	3.2 ± 4.2
%TEP-C	1.4 ± 1.7	3.1 ± 2.6	1.7 ± 0.8

1: mg l^{-1}; 2: µg l^{-1}; 3: mg xanthan gum eq. l^{-1}; 4: x 10^6 ml^{-1}.

2A.3.5 Particulate organic carbon (POC)

POC concentrations in the suspended particulate matter showed small seasonal trend. The POC concentrations ranged from 270 µg-C l^{-1} (February '99) to 1311 µg-C l^{-1} (April 2000) (**Table 2A.1**). During the entire sampling period, higher POC values were observed in the months of June '98 (1252 µg-C l^{-1}), November '98 (937.3 µg-C l^{-1}), August '99 (1311.2 µg-C l^{-1}) and March 2000 (1113.3 µg-C l^{-1}) (**Fig. 2A.5a**). Seasonally, POC values tend to increase with increase in suspended load (**Table 2A.3**).

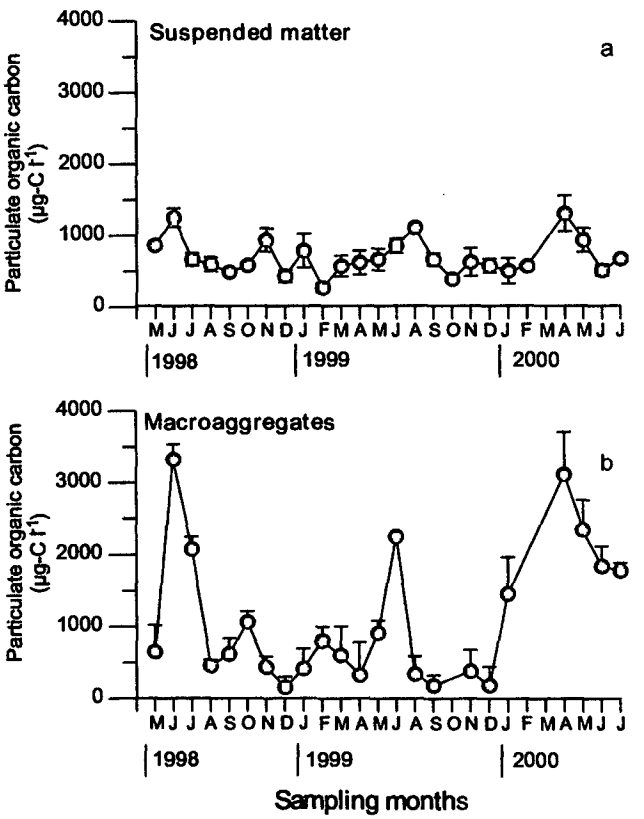

Fig. 2A.5 Seasonal variations in the concentrations of Particulate organic carbon in a) suspended matter and b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

On the other hand, POC concentrations in the macroaggregates showed greater variations during the sampling period. The POC concentrations in the macroaggregates ranged from 156 µg-C l^{-1} (December '98) to a high of 3325 µg-C l^{-1} in the month of June 1998 (**Table 2A.2**). Higher POC values were observed during June '98 (3325 µg-C l^{-1}), October '98 (618 µg-C l^{-1}), July '99 (2250 µg-C l^{-1}) and April 2000 (3108 µg-C l^{-1}) (**Fig. 2A.5b**). The seasonal variations in the POC concentrations in the macroaggregates (**Table 2A.4**) were similar to the POC variations in the suspended particulate matter (**Table 2A.3**).

2A.3.6 Chlorophyll a (Chl a)

The Chl *a* concentrations of the suspended matter showed large scale variations during the study period (**Fig. 2A.6a**). The Chl *a* concentrations ranged from 1.2 µg l^{-1} (July '99) to 12.3 µg l^{-1} (November '98) (**Table 2A.1**). With the onset of monsoon (June-September), an increase in suspended load resulted in lower phytoplankton biomass (3.8 ± 0.9 µg l^{-1}). During post-monsoon (October-January), reduced water column turbidity and higher salinity resulted in higher Chl *a* concentrations (5.9 ± 0.66 µg l^{-1}). Similarly, higher phytoplankton biomass was also observed during pre-monsoon (February-May) months (5.3 ± 2.5 µg l^{-1}) (**Table 2A.3**).

The seasonal variations of Chl *a* concentrations in the macroaggregate samples also showed variations during the sampling period (**Fig. 2A.6b**). Over the period of study, Chl *a* concentrations in the macroaggregates ranged

from 0.4 μg l^{-1} to 6.2 μg l^{-1} **(Table 2A.2)**. Seasonal variations of Chl *a* in the macroaggregates were more prominent in 1998-1999 and the variations matched with the suspended Chl *a* concentrations.

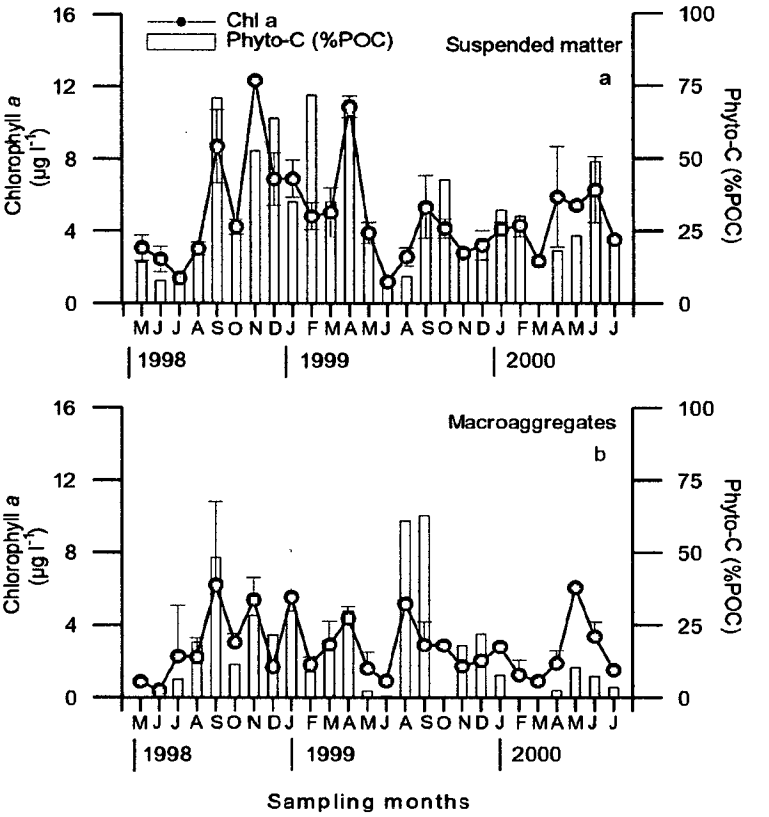

Fig. 2A.6 Seasonal variations in the concentrations of Chlorophyll *a* and Phyto-C (%POC) in a) suspended matter and b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

The phytoplankton carbon (Phyto-C) contribution was estimated using a POC/Chl *a* conversion factor of 40 (Banse 1977). Phyto-C was expressed as % of POC (%Phyto-C) ranged from a low of 5.5% (July '99) to 71.6% (February '99) **(Fig. 2A.6a)**. The average %Phyto-C at the study site was 32% of the average POC (**Table 2A.1**). On the other hand, %Phyto-C in the macroaggregates ranged from a low of 0.4% (June '98) to 62.7% (September

'99) **(Fig. 2A.6b)**. The average Phyto-C in the macroaggregates at the study site was 19.3% of the POC (**Table 2A.2**).

2a.3.7 Total Bacterial Abundance (TBA)

The total bacterial abundance varied from 0.5 x 10^6 (May '99) to 8.2 x 10^6 cells ml^{-1} (December '98) (**Fig. 2A.7a**). Very high abundance of bacteria was observed during post-monsoon months (October-December) in 1998-99 that declined later in the pre-monsoon months (February-May). In the following year, a similar pattern was observed but at a smaller scale.

Bacterial carbon (Bac-C) was estimated using a conversion factor of 11 fg-C $cell^{-1}$ (Garrison et al 2000). Bac-C in the suspended matter ranged from 0.5% (April 2000) to 20.8% (December '99) (**Fig. 2A.7a**). The average contribution of Bac-C to the POC during the entire study period was 4.7% (**Table 2A.1**). The seasonal variation in bacterial carbon indicates maximum contribution during the post-monsoon (October-January) (7.8%) and minimum during the monsoon (June-September) (2.9%) (**Table 2A.3**).

The variations in bacterial abundance in macroaggregates samples were similar to that of suspended particulate matter (**Fig. 2A.7b**). The bacterial abundance varied from 0.5 x 10^6 cells ml^{-1} (February 2000) to 9.5 x 10^6 cells ml^{-1} (December '98). Seasonally, higher bacterial abundance was observed during post-monsoon (October-January) months and lower in pre-monsoon (February-May) months (**Table 2A.4**).

The Bac-C as %POC of the macroaggregates ranged from 0.1% (April '99) to 19.9% (December '99) (**Table 2A.2**). Seasonally, the Bac-C contribution to the POC was greater in 1998-99 than in the following year (**Fig. 2A.7b**). The Bac-C contribution was maximum (6.4%) in post-monsoon and minimum (3.2 %) in pre-monsoon (**Table 2A.4**)

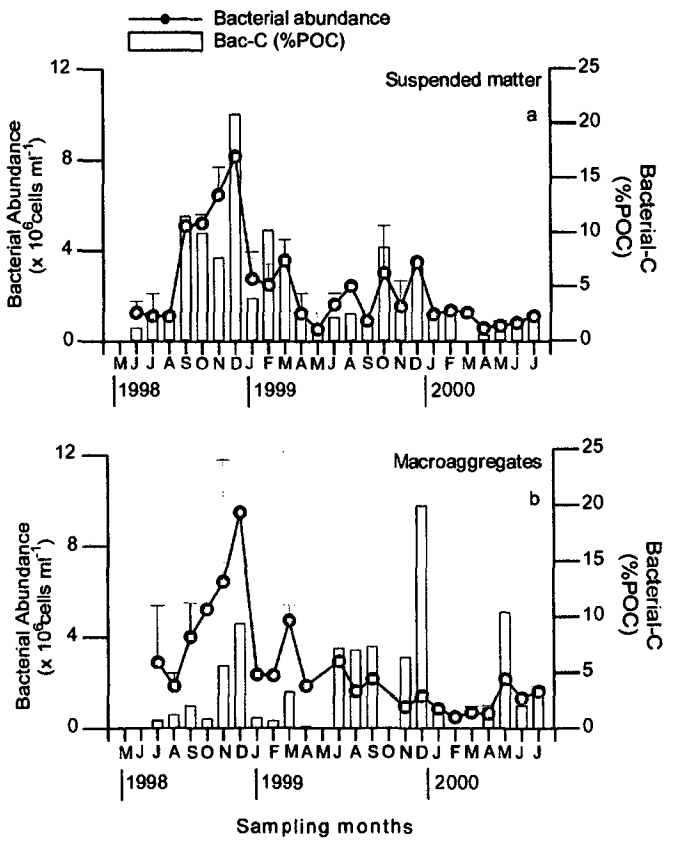

Fig. 2A.7 Seasonal variations in the abundance of total bacteria and bacterial carbon (%POC) in (a) suspended matter and (b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

Bacterial abundance in suspended matter had no significant relation with most of the parameters except for an inverse trend with phytoplankton biomass (r= -0.497) during 1999-2000 (**Table 2A.6**) and moderate linear relationship with TEP concentrations during 1998-1999 (r= 0.748, $p<0.001$) (**Table 2A.5**). In

contrast to suspended matter, the bacterial abundance in the macroaggregate samples had significant inverse relationship with phytoplankton biomass during May 1998 to May 1999 (**Table 2A.7)** and linear relationship with EDTA-soluble and NaCl-soluble EPS during July 1999-2000 (**Table 2A.8).**

2a.3.8 Exopolysaccharides (EPS)

NaCl-soluble EPS and EDTA-soluble EPS

There were clear differences in the distribution of NaCl-soluble EPS concentrations during the sampling period (**Fig. 2A.8a**). The NaCl-soluble EPS concentrations varied from 4.5 μg l^{-1} (March 2000) to 101.6 μg l^{-1} (January '99). The concentrations were minimal in monsoon (June-September) (17.6 μg l^{-1}) and maximal in the post-monsoon (October-January) months (33.8 μg l^{-1}) (**Table 2A.3**). In order to estimate their contribution to POC, NaCl-soluble EPS concentrations were converted to carbon by multiplying with a factor of 0.4. The NaCl-soluble EPS-C concentrations ranged from 0.4% (February 2000) to 5.4% (January '99) (**Fig. 2A.8a**) and the average carbon contribution during the study period was 1.6% of POC (**Table 2A.1)**. The carbon contribution from NaCl-soluble EPS was maximal during post-monsoon months (2.2%) and minimal during monsoon months (1.1%) (**Table 2A.3**).

NaCl-soluble EPS concentrations in macroaggregates varied from 0.9 μg l^{-1} (October '98) to 84.6 μg l^{-1} (July '98) and showed an annual seasonality

during the sampling period (**Fig. 2A.8b**). Seasonally, the NaCl-soluble EPS concentrations were higher during monsoon months and lower during the pre-monsoon months (**Table 2A.4**). In the macroaggregates, the average carbon contribution from NaCl-soluble EPS was 1.7 ± 1.7% and ranged from 0.1 (July 2000) to 6.4% (January '99) (**Table 2A.2**). The variations in NaCl-soluble EPS-C of the macroaggregates showed peaks in the months of January '99 (6.4%), September '99 (3.5%) and November '99 (5.7%) (**Fig. 2A.8b**).

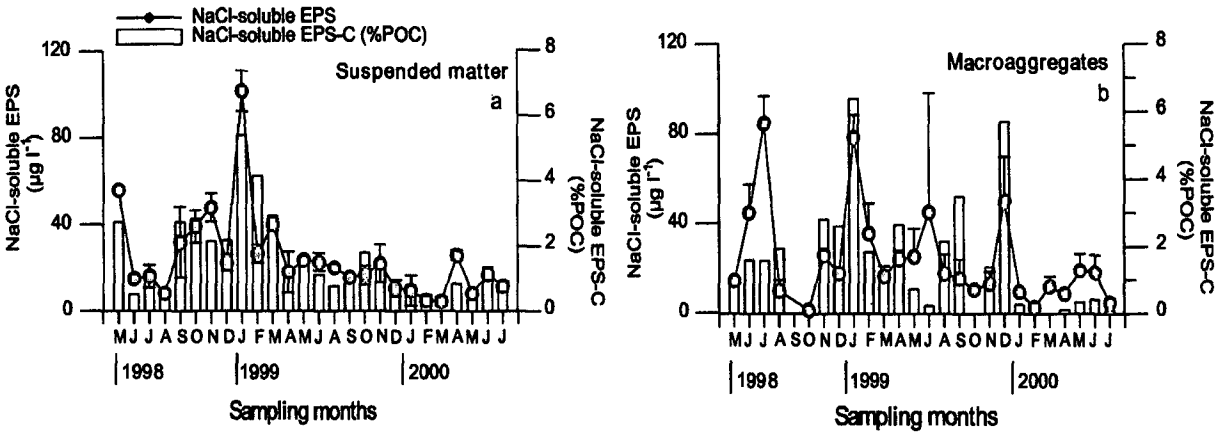

Fig. 2a.8 Seasonal variations in the concentrations of NaCl-soluble EPS and NaCl-soluble EPS-C (%POC) in (a) suspended matter and (b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

Concentrations of EDTA-soluble EPS during the entire study period ranged from 26.2 µg l^{-1} (February '99) to 210 µg l^{-1} (January '99) (**Table 2A.1**). Concentrations of EDTA-soluble EPS did not vary much during May 1998-99 except for the high value in the month of January '99. On the other hand, distinct variations were observed in 1999-2000, with higher concentrations

Table 2A.5. Correlation matrix of various particulate parameters of suspended particulate parameters and nutrients in the year 1998-1999.

Parameters	Chl *a*	POC	EDTA-EPS	NaCl-EPS	TEP	TBA	DO	NO_3	PO_4
Suspended load	-0.533	0.599*	-0.034	-0.080	-0.217	-0.517	-0.220	0.746***	0.271
Chl *a*		-0.081	-0.155	-0.192	0.169	0.242	0.220	-0.556*	0.292
POC			0.100	0.211	0.300	0.174	0.057	0.279	0.144
EDTA-EPS				0.588*	-0.163	0.106	0.079	-0.137	-0.038
NaCl-EPS					-0.123	0.285	-0.242	-0.174	-0.166
TEP						0.748***	0.301	-0.170	-0.200
TBA							0.515*	-0.475	-0.510*

*= p<0.05 & ***= p<0.001

Table 2A.6: Correlation matrix of various particulate parameters of suspended particulate parameters and nutrients in the year 1999-2000.

Parameters	Chl *a*	POC	EDTA-EPS	NaCl-EPS	TEP	TBA	DO	NO_3	PO_4
Suspended load	-0.181	0.217	0.46	0.006	-0.179	-0.236	0.606**	-0.059	0.457
Chl *a*		0.188	0.072	-0.017	0.227	-0.479	-0.189	-0.047	0.304
POC			0.482	0.620**	-0.681**	-0.198	-0.013	-0.311	0.248
EDTA-EPS				0.043	-0.352	-0.153	-0.105	-0.138	0.704***
NaCl-EPS					-0.283	0.008	-0.288	0.071	-0.271
TEP						-0.167	-0.093	0.110	-0.306
TBA							0.083	0.478	-0.288

**= p<0.01 & ***= p<0.001

Table 2A.7. Correlation matrix of various particulate parameters of macroaggregates and nutrients in the year 1998-1999.

Parameters	Chl a	POC	EDTA-EPS	NaCl-EPS	TEP	TBA	DO	NO_3	PO_4
Total macroaggregates	-0.281	0.496	0.379	0.332	-0.046	0.314	-0.266	0.483	0.175
Chl a		-0.472	0.226	0.008	-0.332	-0.610**	0.401	-0.453	-0.083
POC			0.248	0.654**	0.086	0.462	0.063	0.723***	0.115
EDTA-EPS				0.214	-0.165	0.067	-0.174	-0.006	-0.05
NaCl-EPS					0.021	0.107	0.458	0.609	0.122
TEP						0.342	-0.146	0.119	-0.059
TBA							-0.349	0.153	-0.002

**= $p<0.01$ & ***= $p<0.001$

Table 2A.8: Correlation matrix of various particulate parameters of macroaggregates and nutrients in the year 1999-2000.

Parameters	Chl a	POC	EDTA-EPS	NaCl-EPS	TEP	TBA	DO	NO_3	PO_4
Total macroaggregates	-0.126	0.616	0.459	0.755***	0.017	0.563	0.578*	-0.074	0.328
Chl a		-0.059	0.145	-0.169	-0.234	0.284	-0.397	-0.023	0.669**
POC			0.2166	0.0234	0.3339	0.0066	0.405	0.385	0.320
EDTA-EPS				0.4795	-0.5018	0.602*	0.314	-0.246	0.269
NaCl-EPS					-0.2029	0.687**	0.569	-0.056	-0.009
TEP						-0.470	-0.0181	-0.076	-0.331
TBA							0.222	0.362	0.328

*= $p<0.05$, **= $p<0.01$ & ***= $p<0.001$

during monsoon (August '99) and pre-monsoon months (May 2000) (**Fig. 2A.9a**). The %EDTA-soluble EPS-C during the study period ranged from 1.4% (April 2000) to 11.2% (January '99) (**Fig. 2A.9a**). The %EDTA-soluble EPS-C values did not vary much over the seasons (**Table 2A.3**).

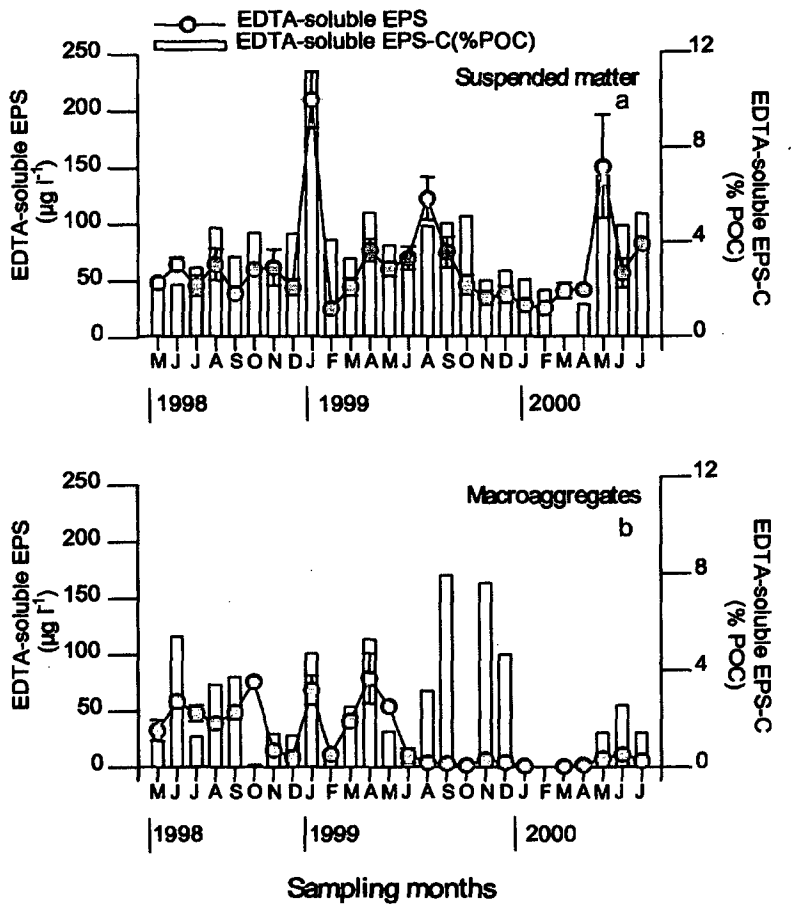

Fig. 2A.9 Seasonal variations in the concentrations of EDTA-soluble EPS and EDTA-soluble EPS-C (%POC) in (a) suspended matter and (b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

The EDTA-soluble EPS concentrations of macroaggregate samples ranged from 0.01 µg l^{-1} (February '99) to 79.2 µg l^{-1} (October '98) and the mean EDTA-soluble EPS concentration was 25.3 ± 26.7 µg l^{-1} (**Table 2A.2**). Distinct

differences were observed in the concentration of EDTA-soluble EPS between 1998-1999 and 1999-2000 (**Fig. 2A.9b**). The mean EDTA-soluble EPS concentrations were similar during monsoon (June-September) (25.7 ± 22.7 μg l^{-1}), post-monsoon (October-January) (22.8 ± 12.3 μg l^{-1}) and pre-monsoon (February-May) season (28.8 ± 30.1 μg l^{-1}). In the macroaggregates, the EDTA-soluble EPS-C as %POC ranged from 0.08% (January 2000) to 7.9% (September '99) during the study period (**Fig. 2A.9b).** The contribution of EDTA-soluble EPS-C was higher during monsoon months and lower during pre-monsoon months (**Table 2A.4**).

The correlation of the two types of EPS was highly variable and behaved differently in the two years. Both NaCl-soluble EPS and EDTA-soluble EPS concentrations in the suspended matter covaried with each other during 1998-1999 ($r= 0.588$; $p<0.05$) but not in 1999-2000 (**Tables 2A.5 & 2A.6**). Moreover, there was no correlation of both forms of EPS with TEP or phytoplankton biomass. In contrast to the suspended particulate matter, NaCl-soluble EPS concentrations in the macroaggregates had no significant correlation with EDTA-soluble EPS during the sampling period (**Table 2A.7 & 2A.8**). Similarly, both forms of EPS did not show any significant correlation with phytoplankton biomass (**Table 2.7 & 2.8**). However, the two forms showed positive linear trends with total macroaggregate concentration and inverse trends with TEP concentrations.

TEP concentrations (Alcian blue method)

TEP concentrations in the suspended matter samples ranged from 1.3 (July '99) to 149 µg Xanthan gum eq. l^{-1} (June 2000) during the entire study period (**Fig. 2A.10a**) and the average concentration was 55.6 ± 35.6 µg Xanthan gum eq. l^{-1} (**Table 2A.1**). There were no seasonal differences in the average

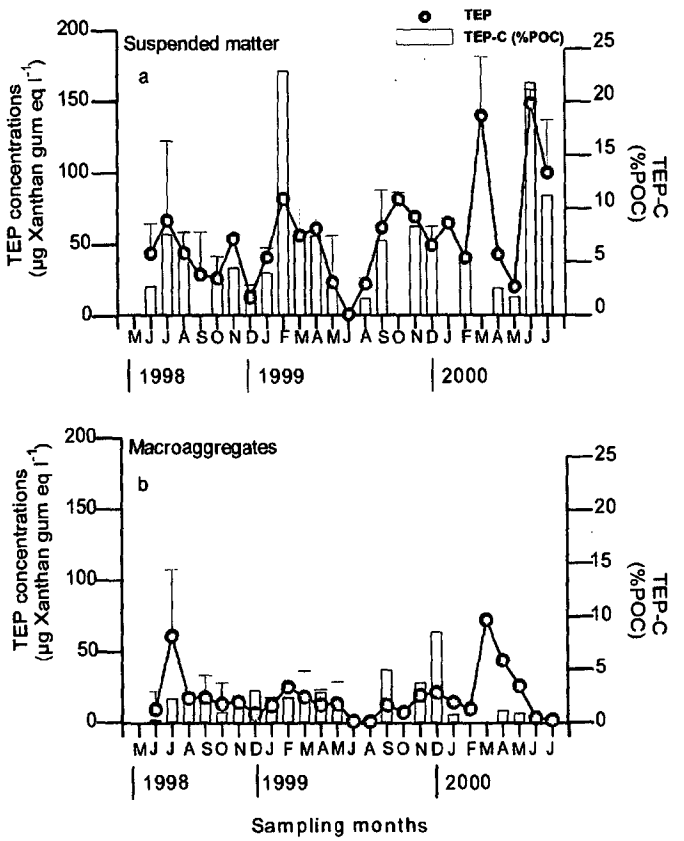

Fig. 2A.10 Seasonal variations in the concentrations of TEP and TEP-C (%POC) in a) suspended matter and b) macroaggregates in the surface waters of Dona Paula Bay during 1998-2000.

TEP concentration for monsoon, post-monsoon and pre-monsoon months (**Table 2A.3**). The TEP-C values expressed as % of POC (%TEP-carbon) ranged from 0.1% (July '99) to 22.8% (February '99) (**Fig. 2A.10a**). The

average TEP-carbon contribution in the suspended matter was 6.9% (**Table 2A.1**) and showed no major seasonal differences (**Table 2A.3**).

TEP concentrations in the macroaggregate samples ranged from 0.4 (August '99) to 72.3 µg Xanthan gum eq. l^{-1} (March '99) (**Table 2A.2**). TEP concentration showed small-scale variations throughout the study period and remained <25 µg Xanthan gum eq. l^{-1} except for the high values observed in July '98 and March '99, respectively (**Fig. 2A.10b**). Seasonally, higher TEP concentrations were observed during pre-monsoon months than in the other two seasons (**Table 2A.4**). The %TEP-C in macroaggregates ranged from 0.01 % (July '99) to 8.5% (December '99) (**Table 2A.2**). The seasonal TEP carbon contribution was greater in the post-monsoon season compared to the other two seasons (**Table 2A.4**).

TEP concentrations in the suspended matter did not have any correlation with phytoplankton biomass and either form of EPS during the sampling period (**Table 2A.3 & 2A.4**). However, TEP had significant linear relation with bacterial abundance ($r=0.748$; $p<0.001$) during 1998-1999 only. On the other hand, TEP concentrations in the macroaggregate samples had no correlation with the other particulate parameters during 1998-99 (**Table 2A.5).** In the following year (1999-2000), TEP had weak inverse trend with EDTA-soluble EPS concentrations (**Table 2A.6**) but no definite trend between TEP concentrations and bacterial abundance.

2A.4 Discussion

EPS and TEP dynamics

Both forms of EPS (NaCl-soluble EPS and EDTA-soluble EPS) in suspended matter and macroaggregates showed temporal and seasonal variations. The concentrations of NaCl-soluble EPS and EDTA-soluble EPS in the suspended matter varied from 4.5 to 101.6 μg l^{-1} and 26.2 to 210.3 μg l^{-1}, respectively. Similarly, NaCl-soluble EPS and EDTA-soluble EPS concentrations in the macroaggregates varied from 0.9 to 84.6 μg l^{-1} and 0.01 to 76.2 μg l^{-1}, respectively. Various factors like phytoplankton biomass, phytoplankton bloom composition, age of the bloom, microbial activity, nutrients and inputs from benthic phytoplankton can influence the concentrations and seasonality of EPS in natural environments (Chrost and Faust 1983, Herndl 1993, Obernosterer and Herndl 1995, Kepkay et al 1997).

Seasonal variations of NaCl-soluble EPS and EDTA-soluble EPS in suspended matter and macroaggregates did not covary with those observed for phytoplankton biomass. The study site experienced higher phytoplankton biomass during the months of September-October, December to February and April (**Fig. 2A.6**). Microscopic observation of the samples revealed that species of *Coscinodiscus, Skeletonema, Nitzschia and Leptocylindrus* constituted bulk of the phytoplankton population during September-October, whereas *Chaetoceros and Nitzschia* were the dominant genera during months of April and June. However, both NaCl-soluble EPS and EDTA-soluble EPS values peaked only in the month of January '99. A similar trend could be observed only for the NaCl-soluble EPS in the macroaggregates during 1998-

99. Our data suggests that the variation in EPS concentrations in suspended matter was not reflected in the macroaggregates. Similarly, higher values of EPS did not match with the higher Chl *a* values in the suspended matter and macroaggregates. This was also evident from the relatively poor relationship between EPS and Chl *a* concentrations (**Tables 2A.5, 2A.6, 2A.7 & 2A.8).** Kepkay et al (1997) observed that production of extracellular compounds like EPS may not always match with Chl *a* concentrations or phytoplankton cell abundance, especially in mixed blooms as observed at the study site. Moreover, the production and nature of EPS produced shall vary with species and the dominant species need not always be the major contributor to EPS in the water column. The variable correlation of EPS with other particulate parameters like suspended load and POC in the water column (**Table 2A.5 and 2A.6**) and total macroaggregates in the macroaggregate samples also indicates that the dynamics of EPS at the study site was not entirely controlled by phytoplankton biomass alone.

Exceptionally high values of NaCl-soluble EPS in both suspended matter and macroaggregates and EDTA-soluble EPS in the suspended matter were observed in January '99. Heavy growth of seaweeds like *Sargassum* was observed at the study site between December to February. It has been reported that brown algae like *Sargassum* release high molecular weight mucus exudates during their growth (Tanaka and Asakawa 1988). It is possible that the mucus released by the seaweeds growing in the study site might have contributed to the exceptionally high EPS concentrations during January '99.

Nutrient concentrations may also play an important role in regulation of EPS concentrations in the water column. During their growth, phytoplankton produces more extracellular organic matter under nutrient-especially nitrate, limiting conditions (Myklestad 1995). Weak inverse trends could be established between nutrients and EPS concentrations in the suspended particulate matter (**Table 2A.3 & 2A.4**). In contrast, dissolved nitrate did not show any inverse trends with NaCl-soluble EPS during the sampling period. On the other hand, EDTA-soluble EPS showed weak negative correlation with nitrate concentrations during 1999-2000 (**Table 2A.5 & 2A.6**). Thus it was evident that the influence of variations in nutrient concentrations on the distribution of EPS was not evident at the study site.

EPS in shallow water stations can come from phytobenthos of the sediments. Since resuspension of bottom sediments is a regular phenomenon in such waters, a close association between EPS and suspended load would be expected. In the present study, suspended load did not have any significant correlation with either NaCl-soluble EPS or EDTA-soluble EPS (**Table 2A.3 & 2A.4**). Thus, neither phytoplankton nor resuspended sediment was a dominant source of EPS in the suspended particulate matter at the study site. On the other hand, EPS in macroaggregates had linear trends with the total macroaggregates (**Table 2A.5 & 2A.6**). From the total macroaggregate data (**Fig 2A.4b)**, it was clear that total macroaggregate concentrations were higher during the monsoon months (June to September). Thus it is possible that resuspended particulate matter might contribute to the total

macroaggregate concentrations. Moreover, the poor correlation between EPS and Chl *a* concentrations suggest that EPS in the macroaggregates was not of phytoplankton origin. Since the station is very shallow and close to the shore, its possible that EPS in the macroaggregates might have alternate sources including benthic inputs.

Another important factor that might regulate EPS dynamics is microbial activity. Both laboratory (Smith et al 1995, Goto et al 2001) and field studies (Reibesell 1991, Janse et al 1999) have shown that bacterial activity can hydrolyze EPS to meet their carbon demand. The total bacterial abundance in the suspended matter did not show any correlation with either NaCl-soluble EPS or EDTA-soluble EPS concentrations (**Table 2A.3 & 2A.4**). In contrast, EPS had a better correlation with bacterial abundance in macroaggregates during 1999-2000 **(Table 2A.6)**. Macroaggregates are enriched in organic matter like EPS, phytoplankton cells etc. (Herndl 1993). EPS adsorbed to macroaggregates might be actively degraded by particle-associated bacteria (Karner and Herndl 1992, Agis et al 1998, Grossart and Simon 1998, Unanue et al 1998, Worm and Sondergaard 1998). Moreover, a tight coupling between the organic matter and the bacterial abundance has been reported in macroaggregates (Goldman 1984). The significant linear correlations in the macroaggregates (**Table 2A.6**) suggest that accumulation of EPS appeared to support bacterial growth. On the other hand, the difference in correlations of EPS and bacterial abundance between 1998-1999 and 1999-2000 might be due to differences in composition of bacterial community and quality of the EPS (Herndl 1993, Decho and Herndl 1995).

In the natural environments, increase in the production of EPS and TEP coincide with termination of phytoplankton blooms (Passow et al 1994). However, a similar phenomenon was not evident at the study site. In the present study, TEP concentrations in the water column were generally higher during the monsoon and pre-monsoon months during which the phytoplankton biomass was relatively low (**Table 2A.1**). The concentration of TEP in shallow water environments might be regulated by various factors including phytoplankton biomass and bacterial activity (Alldredge and Passow 1993, Mari and Kiørboe 1996), land run-off, terrestrial inputs and resuspended sediments (Jahmlich et al 1998, Passow 2002). The higher TEP concentrations during monsoon months might have been due to an increase in resuspended sediments. However, inverse trends between TEP and suspended load indicate that suspended load did not significantly influence the TEP concentrations. On the other hand, fresh water runoff brings in organic matter in the study area, which leads to increase in both dissolved and particulate organic carbon (Qasim and SenGupta 1981). Moreover, TEP concentrations had only a weak positive trend with Chl *a* concentrations (**Table 2A.5 & 2A.6**) and weak negative correlation with EPS (both NaCl-soluble and EDTA-soluble). A similar correlation is also observed in the macroaggregate data (**Table 2A.7 & 2A.8**). Microbial EPS, dissolved organic carbon (DOC) and cell lysate may act as a source of TEP in the aquatic environments (Herndl 1995, Alldredge and Jackson 1995, Passow 2002). However in shallow coastal waters like Dona Paula Bay, additional inputs from allochthonous sources might also play an equally important role in TEP

distribution as observed from the variable correlation of TEP concentrations with EPS and Chl *a* in both the suspended matter and macroaggregates.

TEP are rich in polysaccharides and hence may act as a carbon source for bacteria. In the surface water samples, bacterial abundance had linear correlation with TEP only in the year 1998-99 **(Table 2A.3)**. In the following year, the relation was insignificant and inverse. Such a mixed relation could not be explained using the available data. On the other hand, a weak positive correlation was also observed between TEP concentrations and bacterial abundance in the macroaggregates (**Table 2A.5 & 2A.6**). Such correlations in the suspended matter and macroaggregate samples suggest that increase in TEP might be the result of bacterial activity in the water column. If TEP were to serve as substrate for bacterial growth, the TEP concentration should have decreased with increasing bacterial abundance. However, such a relationship was not evident at the study site. Moreover, a poor correlation was observed between TEP concentrations and bacterial production and extracellular glucosidase activity during 1999-2000 (Chapter 2b). Thus, it appears that TEP at the sampling site might be inert to microbial degradation in the Dona Paula bay.

Contribution of EPS-C to the POC

In the productive coastal waters, phytoplankton followed by bacteria, EPS and TEP together form a major source of POC. Earlier studies in the Dona Paula Bay have shown that extracellular organic carbon released during

phytoplankton blooms contributed about 4 – 38% of the primary production (Gomes et al, 1991). The estimates of EDTA-soluble EPS -C, NaCl-soluble EPS -C and TEP-C contribution to the total POC in the surface waters indicate that TEP-C (6.9%) was second major contributor to the POC after phytoplankton-C. On the other hand, NaCl-soluble EPS-C and EDTA-soluble EPS-C contribution to the POC was 1.7% and 4%, respectively. Assuming 80% removal of EPS by bacteria (Gomes et al 1991), the sum of actual EPS-C contribution would be ~9% of POC. Thus, TEP-C contribution was relatively higher than the sum of the contribution from EDTA-soluble EPS-C (4%) and NaCl-soluble EPS-C (1.7%) and Bact-C (4.7%). On the other hand, the average TEP-C contribution was 21% of the average Phyto-C (32.2% of POC). In comparison to suspended matter, TEP-C in macroaggregates was much less but still greater than %NaCl-soluble EPS-C of the aggregates. However, there was little difference between TEP-C and total carbon contribution of EPS. It is interesting to note that TEP-C did not appear to support bacterial biomass. Although %TEP-C was not very high, this fraction might act as a sink of organic carbon in these waters. However, further studies need to be carried out to ascertain the fate of TEP-C in these waters.

2A.5 Conclusions

A distinct seasonal variation in the concentrations of EPS and TEP was observed in both suspended particulate matter and macroaggregate samples collected from Dona Paula Bay. The concentrations of EPS and TEP and abundance of TEP in both suspended matter and macroaggregates were

controlled by both physical factors (water column turbidity) and biological factors (phytoplankton biomass and bacterial abundance). EPS (except TEP) was not a major source of POC for bacterial population in the study area. The TEP-C was relatively higher than both EPS-C and bacterial carbon in the surface waters of the Dona Paula Bay. However, no relation could be established between bacterial abundance and TEP in the water column. This implies that TEP might not be a preferred carbon source for bacteria in the Dona Paula Bay.

CHAPTER 2B

Ecto-glucosidases activity and bacterial production in the Dona Paula Bay

Chapter 2B

2B. 1 Introduction

Estuaries are highly dynamic and complex ecosystems that are in a constant state of flux. The organic carbon demand in these ecosystems is met by combination of autochthonous and allochthonous sources (Peduzzi and Herndl 1992, Mann and Wetzel 1998, Conan et al 1999) and microbial activity (Fuhrman and Azam 1982, Strom et al 1997). Bacterioplankton growing in such a dynamic environment have to cope with wide fluctuations in physical (tides, salinity, temperature, turbidity etc.) (Shiah and Ducklow 1994, Jorgensen et al 1998, Wikner et al 1999, Cunha et al 2000, Almeida et al 2001) and chemical characteristics (substrate availability, substrate complexity) (Thingstad and Billen 1994, Amon and Benner 1996, Foreman et al 1998, Covert and Moran 2001) in the water column. Moreover, such variations influence the quality and quantity of the organic matter in the water column (Raymond and Bauer 2000, Sonderegaard et al 2000). Such fluctuations may bring about a shift in bacterial production, activity and community structure (Janse 2000, Simon et al 2002).

One of the notable features of bacterial utilization of organic matter (both particulate and dissolved) is the production of an array of ecto-enzymes including glucosidases, peptidases and proteases, which breakdown the complex organic molecules to smaller molecules. These smaller molecules

are then utilized microorganisms for their growth (Chrost, 1989). Due to their substrate specificity, enzymes have also been used to assess the quality of the organic matter in the aquatic environments. For example, glucosidases are carbohydrate specific enzymes that cleave α- or β- 1-6 glycosidic linkages (Somville 1988) and are potentially useful to assess the quality of organic matter in the aquatic environments (Herndl 1993, Decho and Herndl 1995).

Enzyme activities have been measured either by estimating the rate of accumulation of product (Duddridge and Wainwright 1982) and the use of chromophores (Meyer-Reil 1981) or fluorogenic substrate analogs (Hoppe 1983). Fluorogenic substrate analogs are preferred for its sensitivity, enzyme specificity and accuracy and short period of incubation. These substrates have been used widely to assess the heterotrophic activity in freshwater (Middelboe and Søndergaard 1995), limnic (Munster 1991, Grossart and Simon 1998), brackishwater (Hoppe 1983), estuarine and coastal water (Ammerman 1991, Somville 1993, Almeida et al 2001, Cunha et al 2001) and pelagic environments (Cho and Azam 1988, Muller-Niklas et al 1994).

Numerous studies have been carried out to assess the enzyme activity, substrate turnover and bacterial production in oceanic (Muller-Niklas et al 1994, Christian and Karl 1995, Nausch et al 1998), estuarine (Almeida et al 2001, Cunha et al 2001), limnic (Chrost 1991, Vrba 1992, Middleboe and Sondergaard 1993) and riverine (Berger et al 1995) ecosystems of temperate waters. Although tropical coastal waters are one of the most productive

ecosystems, not much is known about bacterial abundance, production and enzyme activity in these ecosystems (Rath et al 1993).

Dona Paula Bay experiences wide fluctuations in the hydrography, nutrients and quality of organic matter (Bhaskar et al 2000). In Chapter 2a, it was shown that both autochthonous inputs like phytoplankton biomass and EPS associated with particulate matter had moderate to significant correlation with bacterial abundance, especially in the macroaggregate samples. The study area is also influenced by detrital inputs from mangroves (DOM and POM) that might support microbial carbon demand (Wafar et al 1997). Keeping in mind the above facts, a 13-month field sampling was carried out during 1999-2000 at a shallow water station in the Dona Paula Bay. The objectives were to study 1) the seasonal variation of bacterial production and ecto-enzyme activity and 2) to assess the role of organic substrates (phytoplankton biomass and EPS) on the heterotrophic activity of bacteria in seawater samples and macroaggregates.

2B. 2 Materials and Methods

Sample collection and analyses

During the year 1999-2000, seawater samples were collected as described in Chapter 2a for the analyses of hydrography (temperature, salinity) and nutrients (nitrate and phosphates). Macroaggregate samples were also collected following the method described in Chapter 2a. Both seawater and macroaggregate samples were then filtered onto preashed GF/F filters for the

analyses of various particulate parameters like dry weight, Chl *a*, POC, EPS and TEP. Sub-samples of seawater and macroaggregates were fixed with 0.22 µm filtered formalin (4% final concentration) for bacterial enumeration. Suitable sub-samples were also collected to estimate bacterial production and enzyme activity. Methods for estimation of temperature, salinity, nutrients and the particulate parameters were same as described in Chapter 2a except for bacterial production and enzyme activity, the details of which are described below.

2B.2.1 Bacterial abundance

The formalin fixed seawater and macroaggregate samples were filtered onto 0.22 µm prestained Nuclepore membrane filters and stained with 0.22 µm pre-filtered acridine orange for the enumeration of total bacterial abundance following Parsons et al (1984) as given in Chapter 2a. Additional samples were filtered through GF/F filters and the filtrate was collected in sterile glass tubes. One ml of the filtrate containing particle free bacteria, was then stained with acridine orange, filtered onto 0.22 µm prestained Nuclepore membrane filters and enumerated as described in Chapter 2a. Particle associated bacteria (PAB) was calculated as the difference between total and particle free bacterial population.

2B.2.2 Bacterial production

Working solution of thymidine

A working solution of $^{3}H_{1}$ –thymidine was prepared by drying one ml of $^{3}H_{1}$–thymidine stock solution (1mCi; Sp. activity: 12000 mCi $mmole^{-1}$) under a stream of nitrogen. The dried thymidine was then diluted in 3.3 ml deionized water to obtain a working solution of 5 nmole ml^{-1} thymidine concentration.

Methodology

The bacterial production rates in the seawater and macroaggregate samples were estimated from the incorporation rates of $^{3}H_{1}$-thymidine. The samples were analyzed following the JGOFS protocol (1994). In order to estimate total bacterial production, ten ml of unfiltered seawater and macroaggregates samples in triplicate were dispensed in test-tubes. Twenty µl working solution of thymidine was added (10 nM thymidine final concentration) to the test-tubes. Similarly, one set of sample was fixed with filtered formalin (4% final concentration) prior to addition of $^{3}H_{1}$-thymidine and treated as control. Both sets were incubated in dark for 60 mins at room temperature. In order to estimate the particle free bacterial production, 10 ml of both seawater and macroaggregate samples filtered through preashed GF/F filters at <10 mbar pressure, was inoculated with 20 µl of $^{3}H_{1}$-thymidine and estimated for particle free bacterial production as mentioned above.

At the end of incubation period, uptake of $^{3}H_{1}$-thymidine was terminated with the addition of 0.22 µm filtered formalin (4% final concentration). The samples were filtered at <10 mbar pressure through 0.22 µm cellulose nitrate Millipore filters pretreated with ice-cold 5% trichloroacetic acid (TCA). The filter papers were then rinsed thrice with 5% ice-cold TCA, followed by three rinses of ice-cold ethanol and were filtered to dryness. The filter paper was then transferred to clean scintillation vials and completely dissolved in 2 ml ethyl acetate. Four ml of scintillation cocktail was added to each sample and allowed to stabilize overnight. The replicate samples were counted over one minute using a scintillation counter (Wallac 2000).

The thymidine uptake rate was calculated using the following equation:

$$[\text{methyl-}^{3}\text{H-thymidine}]\ \text{nM h}^{-1} = \frac{(dpm_{sample} - dpm_{blank})}{2200} \times \frac{1000}{V} \times \frac{1}{SA} \times \frac{60}{T}$$

where dpm is disintegrations per minute; 2200 is dpm per nCi; V is extraction volume, SA is the specific activity and T is incubation time (hours).

The thymidine uptake rate was converted to bacterial production (Bp) using a conversion factor (CF) of 2 x 10^{18} cells per mmole of thymidine utilised (Riemann et al. 1987) as given below:

Bacterial production (Bp) (µg-C l^{-1} h^{-1})= Thymidine counts x CF x carbon $cell^{-1}$;

where carbon $cell^{-1}$ was assumed to be 11 femto g (Garrison et al 1999). Cell-specific production (SBp) was calculated as below:

$$SBp\ (\text{femto g-C cell}^{-1}\ h^{-1}) = \frac{\text{Bacterial production}}{\text{Bacterial abundance}}$$

2B.2.3 Ecto-glucosidase activity

Working solution of MUF-substrates

The working solution was prepared using 1:1 dilution of 100 µM substrate analogs (4-methyl umbelliferryl (MUF) α- and β-glucoside) stock solution (33.8 $\mu g\ ml^{-1}$) in deionized water. The substrate standard stock was kept frozen at –20°C and fresh working solution was prepared before each sample analyses.

The ecto-glucosidase activity in the seawater and macroaggregate samples was estimated using (MUF) α- and β-glucosides (Hoppe 1983 & 1993). The fluorescent substrate analogs are substrates that are linked to artificial fluorochromes by specific bindings. When added to a natural sample, these substrates are enzymatically cleaved and the amount of fluorochromes released is used as a direct measure of the microbial enzyme activity.

Calibration

The method was calibrated for the required substrate concentration and incubation period for both MUF-α and β-glucoside. Briefly, substrate solution of increasing concentrations (5, 10 and 20 nM final concentration) were added to unfiltered seawater samples in triplicate and incubated at room temperature (28 ± 2°C) up to 10 h. Sub-samples were removed after every 2 h and the fluorescence of the substrate was measured. Calibration of fluorochrome was carried out using increasing concentrations of MUF (methyl umbelliferone) standard (2, 4, 6, 8, 10 & 20nM). A factor was calculated by plotting the fluorescence intensity against the concentration and was later used in the calculation of the enzyme activity.

Experimental setup

In order to estimate the total ecto-glucosidase activity (both α- and β-glc), 5 µl (50µM) MUF-α & β glucoside was added to 25 ml of surface seawater and macroaggregate samples (10 nM final concentration) dispensed into 50 ml Ehrlenmeyer flasks. Similarly, 5 µl of 50µM MUF-α & β glucoside was added to 25 ml of GF/F filtered surface seawater and macroaggregates dispensed in 50 ml Ehrlenmeyer flasks to estimate the particle free ecto-glucosidase activity. The particle associated ecto-glucosidase activity was calculated as the difference between total and particle free glucosidase activity. In order to estimate the dissolved cell-free glucosidase activity, MUF-substrates were

added to seawater samples filtered through 0.22 µm cellulose nitrate filters. One set of unfiltered samples was heat killed (100°C) prior to addition of MUF-substrate and used as blank. Both the samples and blanks were incubated in dark at room temperature (28 ± 2°C) for a period of 2 to 5 h.

After incubation, 1ml of the sample was transferred into a cuvette containing 100 µl of Tris-HCl buffer (pH 10.3), mixed properly and measured at 365 nm excitation and 460 nm emission wavelengths using a fluorescence spectrophotometer (Hitachi F-2000).

The rate of enzyme activity was calculated as follows:

$nM\ C\ h^{-1} = (f_t - f_0) \times F \times$ carbon content of the substrate;

where $f_t - f_0$ is the difference in fluorescence between final time t_f and incubation time t_0; F is the standard factor (Standard concentration/Standard fluorescence) obtained from calibration curve and carbon content was 72 µg C μM^{-1} (Hoppe, 1993). The cell-specific activity (femto mol-C $cell^{-1}\ h^{-1}$) was calculated as below:

$$\text{Cell-specific activity (Sp- activity)} = \frac{\text{Total enzyme activity}}{\text{Total bacterial Abundance}}$$

2B.2.4 Statistical Analyses

Pearson correlation coefficients were obtained between various particulate parameters and bacterial abundance, bacterial production and enzyme

activity in both seawater samples and macroaggregate samples. In order to assess the influence of the particulate matter composition on the microbial activity in the macroaggregates, correlations were obtained between the bacterial production and enzyme activity of macroaggregate samples with various particulate parameters of the seawater samples. The closeness of the particulate parameters on the microbial activity was further evaluated using Principal component analysis method after extracting the factor loadings.

2B.3 Results

2B.3.1 Calibration

The calibration for optimal incubation time and optimal substrate concentrations required to estimate the ecto-glucosidase activity is shown in **Fig. 2B.1a** and **Fig. 2B.1b**, respectively. It was found that substrate concentration of 10 nM and an incubation time of 2 h was optimal. These optimal conditions were employed to evaluate the enzyme activity at the Dona Paula Bay.

2B.3.2 Bacterial Abundance

Total bacterial abundance (TBA) in the seawater samples varied from 0.6 x 10^6 cells ml^{-1} (April 2000) to 3.5 x 10^6 cells ml^{-1} (December '99) during the

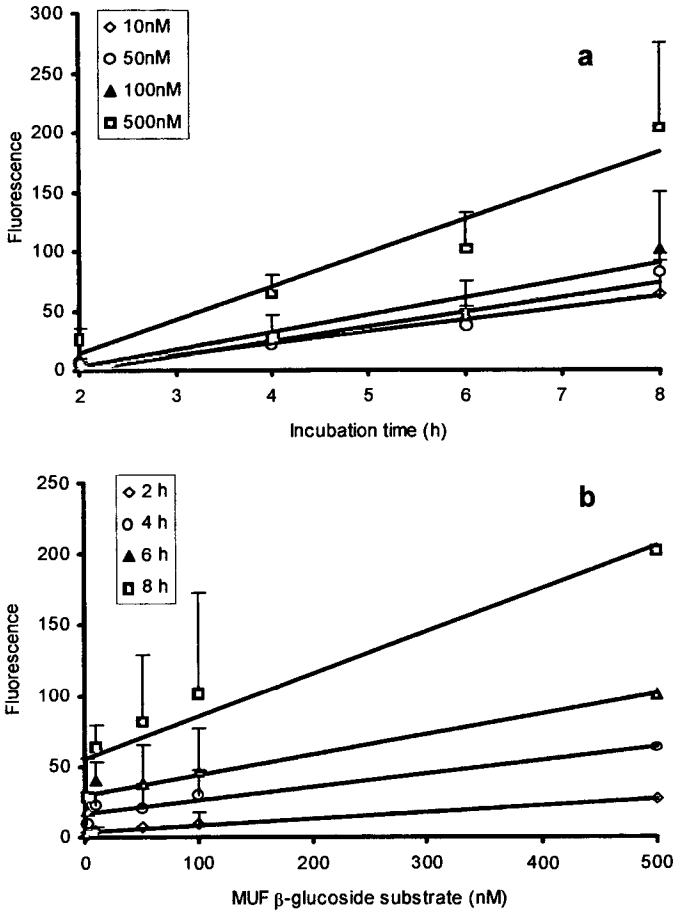

Fig. 2B.1 Linear plots showing the effect of incubation time (a) and MUF-substrate concentrations (b) on b-glucosidase activity. A substrate concentration of 10 nM (r^2= 0.994) with an incubation period of 2 hours (r^2= 0.978) was optimum to estimate the enzyme activity.

sampling period (**Fig. 2B.2a**). TBA showed an increasing trend from July to December '99 followed by a sharp decline for the remaining period of sampling.

The total bacterial abundance in macroaggregates varied from 0.5 x 10^6 cells ml^{-1} (February 2000) to 2.9 x 10^6 cells ml^{-1} (July '99) during the sampling period (**Fig. 2B.2b**). The bacterial abundance declined from July '99 until February 2000 followed by a small increase for the remaining period of observation.

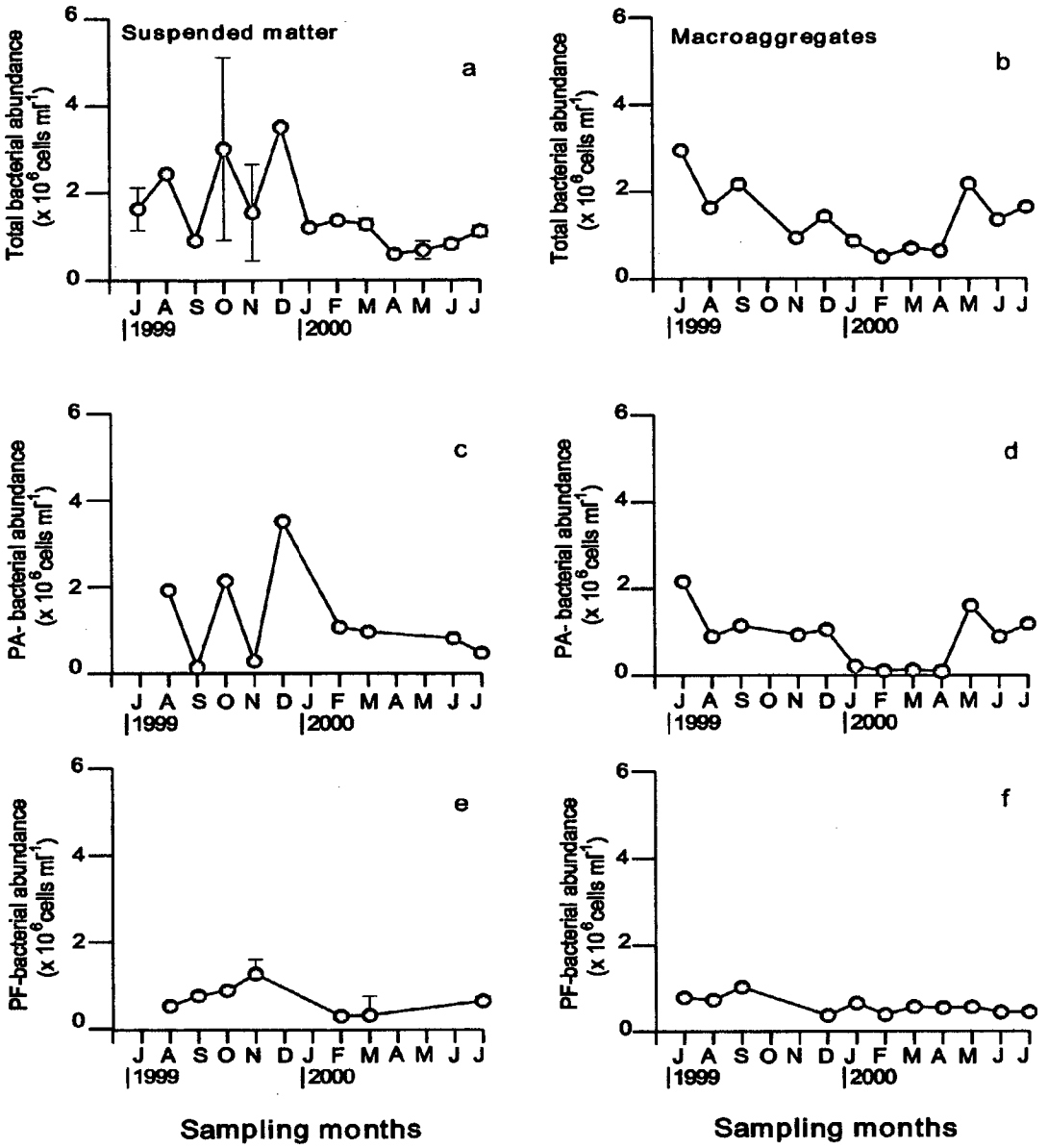

Figure 2B.2. Temporal variations of Total bacterial abundance (a & b), Particle associated bacterial abundance (c & d) and Particle Free-living Bacterial abundance (e & f) in the suspended matter and macroaggregate samples collected from Dona Paula Bay during 1999-2000. Each point is the average of duplicate counts and the error-bars indicate SD.

Particle associated bacterial abundance (PAB) in the seawater samples ranged from 0.15 x 10^6 cells ml^{-1} (September '99) to 3.4 x 10^6 cells ml^{-1} (December '99) **(Fig. 2B.2c).** On the other hand, PAB in macroaggregates

ranged from 0.08 x 10^6 cells ml^{-1} (April 2000) to 2.2 x 10^6 cells ml^{-1} (July '99), respectively (**Fig. 2B.2d**). On an average, particle associated bacteria in seawater and macroaggregate sample was 1.3 ± 1.1 x 10^6 cells ml^{-1} and 0.87 ± 0.65 x 10^6 cells ml^{-1} (**Table 2B.1 and 2B.2**), which accounted for 49% and 61% of the total bacterial abundance, respectively. Particle free bacterial (PFB) abundance in seawater and macroaggregate samples varied from 0.3 to 1.3 x 10^6 cells ml^{-1} (**Fig. 2B.2e**) and 0.37 to 1.02 x 10^6 cells ml^{-1} (**Fig. 2B.2f**) during the sampling period. Moreover, the counts were relatively higher in 1999 than in 2000. However, no specific trend was observed in the seasonality of the PFB in both suspended matter and macroaggregate samples.

2B.3.3 Bacterial production

The total bacterial production (TBp) ranged from 0.4 µg-C $l^{-1}h^{-1}$ (November '99) to 3.2 µg-C $l^{-1}h^{-1}$ (June 2000) (**Fig. 2B.3a)** and showed large-scale variations without any definite trend during the sampling period. TBp increased from 0.5 µg-C $l^{-1}h^{-1}$ (August '99) to 3.1 µg-C $l^{-1}h^{-1}$ (September '99), followed by a similar higher bacterial production during the months of December '99 (2 µg-C $l^{-1}h^{-1}$) and June 2000 (3.2 µg-C $l^{-1}h^{-1}$) (**Fig. 2B.3a**). The specific bacterial productivity (SBp) ranged from 0.3 femto g-C $cell^{-1}h^{-1}$ (May 2000) to 2.6 femto g-C $cell^{-1}h^{-1}$ (April 2000) and the average specific production was 1.2 ± 0.8 femto g-C $cell^{-1}h^{-1}$ during the entire sampling period (**Table 2B.1**).

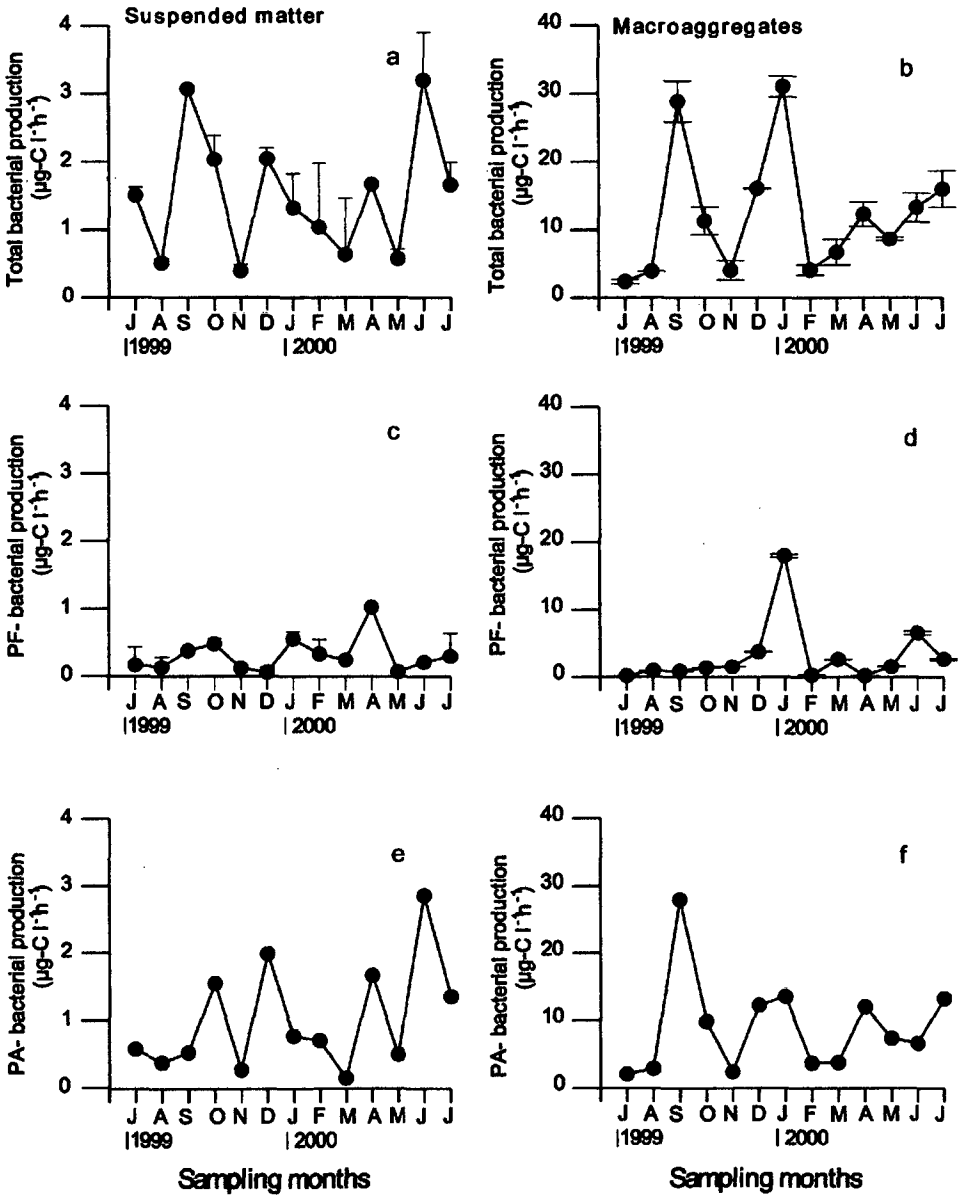

Figure 2B.3. Temporal variations of Total bacterial production, Particle Free bacterial production and Particle Associated Bacterial production in the suspended matter (a, c & e) and macroaggregates (b, d & f) in the surface waters of Dona Paula Bay during 1999-2000. Each point is mean of duplicate samples and the error-bars indicate SD.

Table 2B.1: Mean, Sd and range of bacterial abundance, production, ecto-glucosidase activity, specific bacterial production, specific enzyme activity and enzyme ratio in the seawater samples during 1999-2000.

Parameters		Mean	SD	Max	Minimum
TBA	x 10^6 cells ml^{-1}	2.40	1.99	2.95	0.83
PAB	x 10^6 cells ml^{-1}	1.00	0.61	2.17	0.08
TBp	µg-C l^{-1} h^{-1}	1.60	0.99	3.60	0.39
PABp	µg-C l^{-1} h^{-1}	1.20	0.95	3.07	0.27
SBp	femto g-C $cell^{-1}$ h^{-1}	1.20	0.80	2.64	0.26
Total α-glcA	nM-C h^{-1}	22.60	32.60	127.50	2.28
PA- α-glcA	nM-C h^{-1}	19.20	26.19	101.44	1.59
PF- α-glcA	nM-C h^{-1}	3.70	7.19	26.07	0.06
Total β-glcA	nM-C h^{-1}	17.00	15.19	62.40	3.03
PA- β-glcA	nM-C h^{-1}	13.80	10.42	39.80	3.03
PF- β-glcA	nM-C h^{-1}	4.20	6.73	22.60	0.08
α/β ratio		1.10	0.44	2.04	0.50
Sp-α-glcA activity	femto mol $cell^{-1}$ h^{-1}	14.70	15.00	58.73	10.51
Sp-β-glcA activity	femto mol $cell^{-1}$ h^{-1}	12.40	9.10	29.40	2.11

TBA: Total bacterial abundance; **PAB**: Particle associated bacterial abundance; **TBp**: Total bacterial production; **PABp**: Particle-associated bacterial production; **SBp**: Specific bacterial production; **α-glcA**: α-glucosidase activity; **β-glc A**: β-glucosidase activity; **PF-activity**: Particle free activity; **Sp-activity**: Specific activity

TBp in the macroaggregates ranged from 2.3 µg-C $l^{-1}h^{-1}$ (July '99) to 31 µg-C l^{-1} h^{-1} (January 2000) (**Fig. 2B.3b**). The TBp increased from 2.3 µg-C l^{-1} h^{-1} (July '99) to 28.8 µg-C l^{-1} h^{-1} (September '99) and declined thereafter to 4 µg-C l^{-1} h^{-1} (November '99). Thereafter, a production maximal was observed in January 2000 (3.2 µg-C l^{-1} h^{-1}), declined in February 2000 (4 µg-C l^{-1} h^{-1}) followed by a steady increase until the end of the sampling period. The values were generally < 20 µg-C l^{-1} h^{-1} except for those recorded in the months of September 1999 and January 2000 (**Fig. 2B.3b**). Moreover, specific

production in the macroaggregates ranged from 2.7 femto g-C $cell^{-1}h^{-1}$ (July '99) to 81.8 femto g-C $cell^{-1}$ h^{-1} (April 2000) (**Table 2B.2**).

Table 2B.2: Mean, Sd and range of bacterial abundance, production, ecto-glucosidase activity, specific bacterial production, specific enzyme activity and enzyme ratio in the macroaggregate samples during 1999-2000.

Parameters		Mean	SD	Max	Minimum
TBA	x 10^6 cells ml^{-1}	0.36	0.19	0.88	0.14
PAB	x 10^6 cells ml^{-1}	0.22	0.17	0.65	0.02
TBp	µg-C l^{-1} h^{-1}	12.20	9.20	31.00	2.30
PABp	µg-C l^{-1} h^{-1}	9.10	7.10	28.0	2.10
SBp	femto g-C $cell^{-1}$ h^{-1}	33.5	23.7	81.8	2.65
Total α-glcA	nM-C l^{-1} h^{-1}	35.1	36.3	116.0	4.60
PA- α-glcA	nM-C l^{-1} h^{-1}	33.0	36.7	114.1	1.10
PF- α-glcA	nM-C l^{-1} h^{-1}	2.10	4.10	15.40	0.00
Total β-glcA	nM-C l^{-1} h^{-1}	31.5	35.7	126.1	2.60
PA- β-glcA	nM-C l^{-1} h^{-1}	28.9	36.4	123.8	2.60
PF- β-glcA	nM-C l^{-1} h^{-1}	0.98	1.90	6.50	0.00
α/β ratio		1.19	0.33	1.75	0.73
SP-α-glcA activity	femto mol $cell^{-1}$ h^{-1}	101.4	93.7	270	20.0
SP-β-glcA activity	femto mol $cell^{-1}$ h^{-1}	87.05	87.1	293	11.5

TBA: Total bacterial abundance; **PAB**: Particle associated bacterial abundance; **TBp**: Total bacterial production; **PABp**: Particle-associated bacterial production; **SBp**: Specific bacterial production; **α-glcA**: α-glucosidase activity; **β-glc A**: β-glucosidase activity; **PF-activity**: Particle free activity; **Sp-activity**: Specific activity

The particle free bacterial production (PFBp) values in the seawater samples varied from 0.06 µg-C l^{-1} h^{-1} (December '99) to 1.03 µg-C l^{-1} h^{-1} (April 2000) (**Fig. 2B.3c**). On the other hand, PFBp in macroaggregates ranged from 0.2 µg-C l^{-1} h^{-1} (July '99) to 18 µg-C l^{-1} h^{-1} (January 2000) (**Fig. 2B.3d**). PFBp in both seawater and macroaggregate samples showed small scale variations.

The trend in seasonal variation of particle associated bacterial production (PABp) was relatively different from TBp in seawater samples. PABp values ranged from 0.15 µg-C $l^{-1}h^{-1}$ (March 2000) to 2.9 µg-C $l^{-1}h^{-1}$ (June 2000) (**Fig. 2B.3e**). The average PABp was 1.2 ± 0.95 µg-C $l^{-1}h^{-1}$, which accounted for 75% of the total bacterial production. During 1999, the PABp was lower between June to September despite higher TBp. Thereafter, PABp showed relatively higher values between October to January and later in the months of April and June 2000. On the other hand, the seasonality observed in PABp of macroaggregates was similar to that of TBp (**Fig 2B.3f).** The PABp values ranged from 2.1 µg-C l^{-1} h^{-1} (July '99) to 28 µg-C l^{-1} h^{-1} (September '99) and the average PABp was 9.1 ± 7.1 µg-C l^{-1} h^{-1}, which constituted 75% of the total bacterial production, which was similar to the PABp in seawater samples.

2B.3.4 Ecto-glucosidase activity

The total α-glc recorded in the seawater samples during 1999-2000 ranged from 2.3 nM-C h^{-1} (March 2000) to 127.5 nM-C h^{-1} (September '99) (**Fig. 2B.4a**). The α-glc activity increased from 19.5 nM-C h^{-1} (July '99) to 127.5 nM-C h^{-1} (September '99), followed by a decline to 2.3 nM-C h^{-1} (March 2000). Thereafter, the activity increased steadily to reach 29.4 nM-C h^{-1} (July 2000). The specific α-glc activity ranged from 6.5 femto mol-C $cell^{-1}$ h^{-1} (August '99) to 58.7 femto mol-C $cell^{-1}$ h^{-1} (September '99) (**Table 2B.1**). The α–glc activity in the macroaggregate samples ranged from 4.6 nM-C h^{-1} (February 2000) to 116 nM-C h^{-1} (September '99) (**Fig. 2B.4b**). The α-glc activity of the

macroaggregates had higher values during September '99 (116 nM-C h^{-1}) and May 2000 (107.7 nM-C h^{-1}) and was lower during November to February (1.1 nM-C h^{-1}; February 2000) . The specific α-glc activity ranged from 20 femto mol-C $cell^{-1}$ h^{-1} (February 2000) to 270 femto mol-C $cell^{-1}$ h^{-1} (September '99) (**Table 2B.2**).

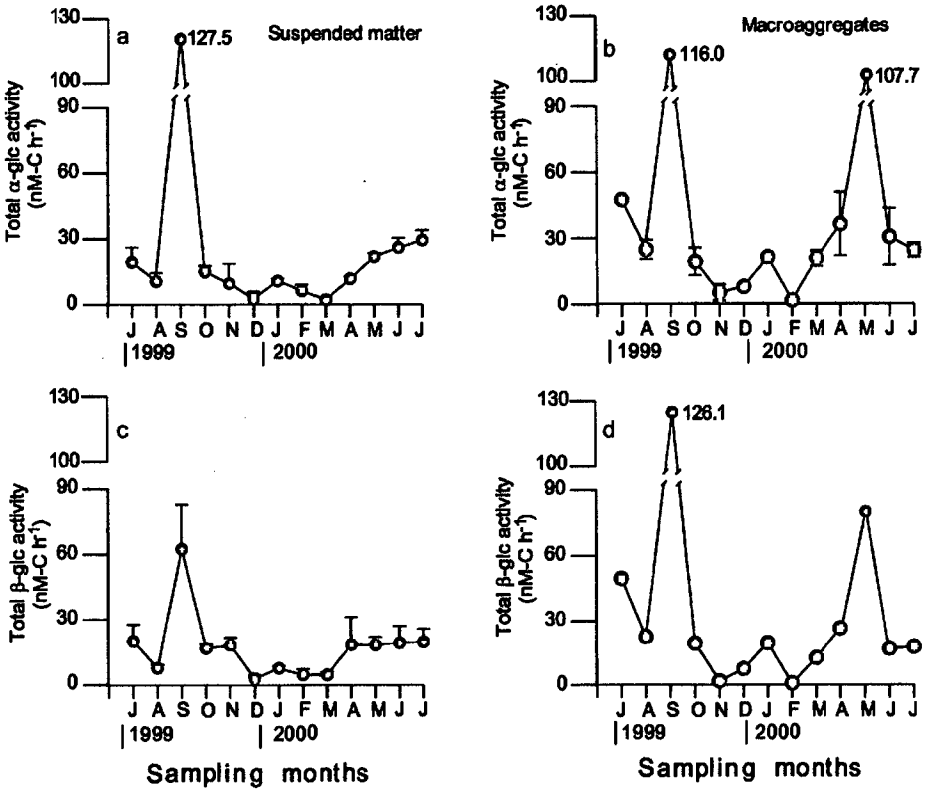

Fig. 2B.4 Seasonal variation of Total α-glc and Total β-glc activity in the suspended matter (a & c) and macroaggregates (b & d) in the Dona Paula Bay during 1999-2000.

The total β-glc activity in the seawater samples during the study period ranged from 3 nM-C h^{-1} (December '99) to 62.4 nM-C h^{-1} (September '99) (**Fig. 2B.4c**). The β-glc activity declined from 20.2 nM-C h^{-1} (July '99) to 7.7 nM-C h^{-1}, followed by an increase to 62.4 nM-C h^{-1} (September '99). Thereafter, the activity decreased to 3 nM-C h^{-1} (December '99), increased to 18.5 nM-C h^{-1}

during April 2000 and did not vary further. The specific β-glc activity ranged from 4.7 femto mol-C $cell^{-1}$ h^{-1} (August '99) to 28.8 femto mol-C $cell^{-1}$ h^{-1} (September '99) (**Table 2B.1**). The total β-glc activity and its seasonality in the macroaggregate samples were similar to that of $\tilde{\alpha}$-glc activity of macroaggregates and ranged from 1.5 nM-C h^{-1} (February 2000) to 126.1 nM-C h^{-1} (September '99) (**Fig. 2B.4d**). The specific β-glc activity in the macroaggregates ranged from 11.5 femto mol-C $cell^{-1}$ h^{-1} (November 2000) to 293 femto mol-C $cell^{-1}$ h^{-1} (May 2000) (**Table 2B.2**).

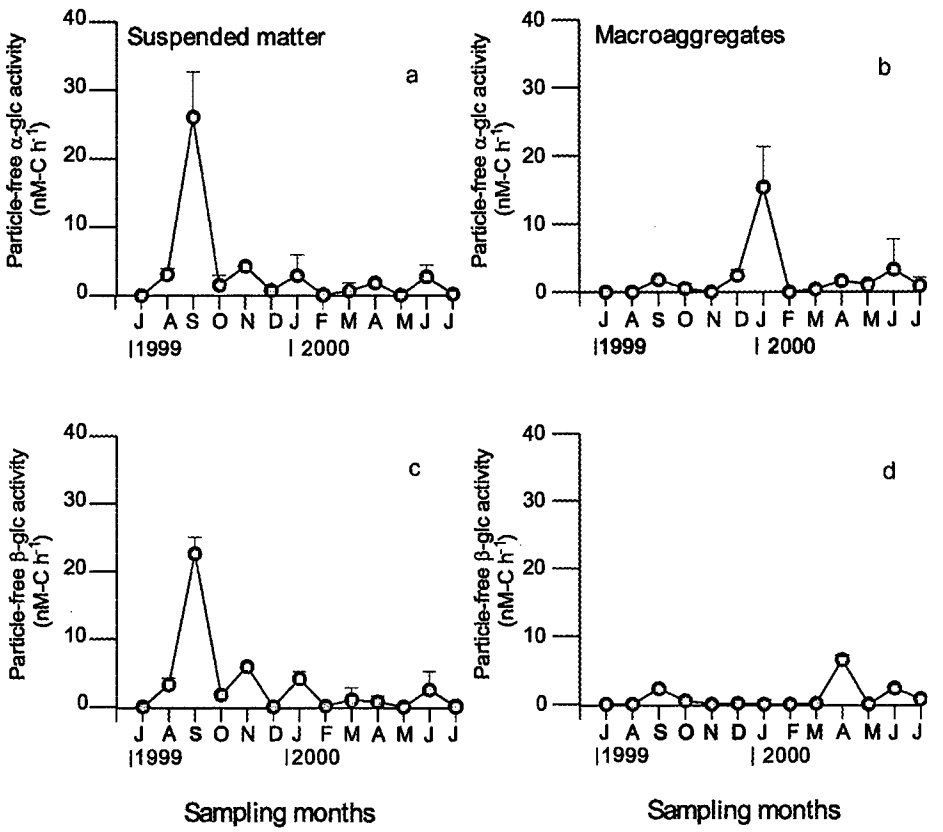

Fig 2B.5 Seasonal variation of Particle-free α-glc and Particle-free β-glc activity in the suspended matter (a & c) and macroaggregates (b & d) in the Dona Paula Bay during 1999-2000.

Except for the high enzyme activity recorded for both particle-free (PF) α-glc (26.1 nM-C h^{-1}) (**Fig. 2B.5a**) and PF β-glc activity (22.6 nM-C h^{-1}) (**Fig. 2B.5b**) in the month of September '99, the seasonal variation of the PF-glc activity in the suspended matter did not have any specific trend and showed small-scale variations. The mean PF- α glc and PF- β glc activity for the entire study period was 3.7 ± 7.2 (16.4% of total α-glc) and 4.2 ± 6.7 nM-C h^{-1} (25% of total β-glc), respectively (**Table 2B.1**). Similarly, except for a high of 15.4 nM-C h^{-1} (January 2000) of PF α-glc activity (**Fig. 2B.5c**) and 6.3 nM-C h^{-1} (April 2000) of β-glc activity in the macroaggregate samples, the samples showed little PF-glc activity (**Fig. 2B.5d**), respectively. The average PF-α̃-glc and PF-β̃-glc activity in the macroaggregates for the entire study period was 2.1 ± 4.1 (6% of total α-glc activity) and 1.0 ± 1.9 nM-C h^{-1} (3.1% of total β-glc activity), respectively.

The particle-associated (PA) glc-activity (α- or β-glc) was calculated as the difference between the total glc and PF-glc activity and constituted a significant fraction of the total glucosidase activity. The temporal variations of PA-glc activity was similar to that of total glc activity in both seawater and macroaggregate samples. The PA-α and PA-β glc activity ranged from 1.6 nM-C h^{-1} (March 2000) to 101.4 nM-C h^{-1} (September '99) and 3 nM-C h^{-1} (December '99) to 39.8 nM-C h^{-1} (September '99), respectively (**Fig. 2B.6a & 2B.6b**). The mean PA-α and β-glc activity in the seawater was 19.2 ± 26.2 nM-C h^{-1} (85% of total α-glc activity) and 13.8 ± 10.4 nM-C h^{-1} (81% of total β-

glc activity), respectively (**Table 2B.1**). The PA– α– and β-glc activity of macroaggregates ranged from 1.1 nM-C h^{-1} (February 2000) to 114.1 nM h^{-1} (September '99) and below detectable limits to 123.8 nM-C h^{-1} (September '99), respectively (**Fig. 2B.6c & 2B.6d**). The mean PA– α– and β-glc activity of macroaggregates was 33 ± 36.7 nM-C h^{-1} (94% of total α-glc activity) and 28.9 ± 36.4 nM-C h^{-1} (92% of total β-glc activity), respectively (**Table 2B.2**). The extracellular (dissolved) glucosidase activity was negligible.

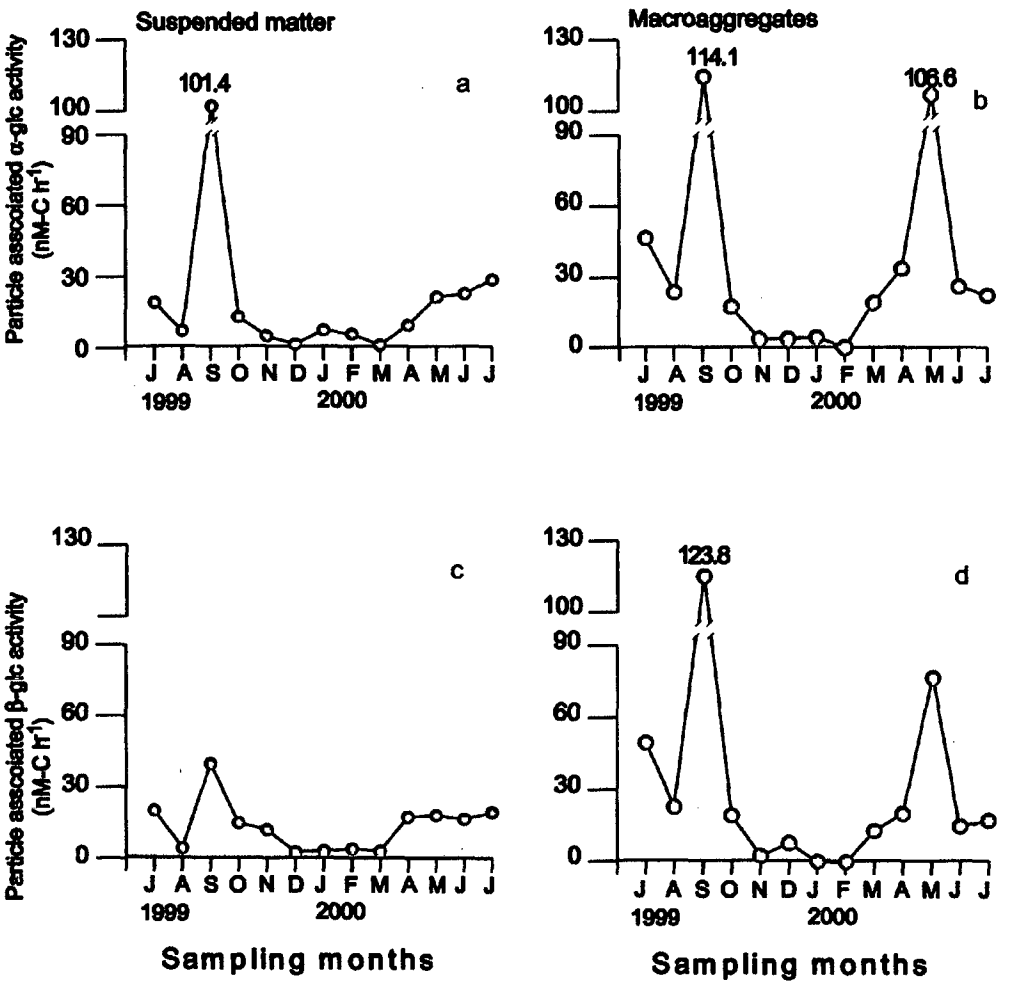

Fig. 2B.6 Seasonal variation of Particle-associated α-glc and Particle associated β-glc activity in the suspended matter (a & c) and macroaggregates (b & d) in the Dona Paula Bay during 1999-2000.

2B.3.5 α-glc/β-glc ratios

The ratios in seawater and macroaggregate samples varied from 0.5 (March 2000) to 2.04 (September '99) and 0.74 (February 2000) to 1.75 (November '99), respectively (**Fig. 2B.7**). The average enzyme ratio in seawater (1.1 ± 0.44) and macroaggregate (1.2 ± 0.33) was similar (**Table 2B.1 & 2B.2**). Interestingly, the ratio in seawater samples showed a trend opposite to that observed for macroaggregates (**Fig. 2B.7**).

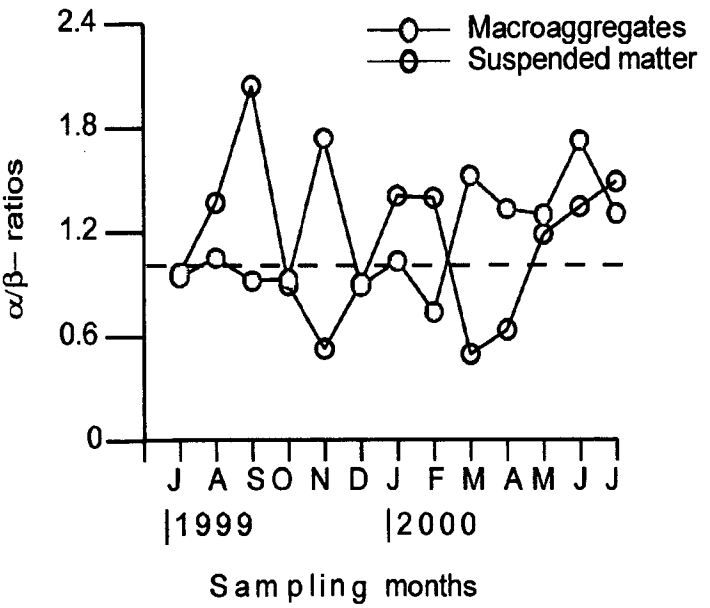

Fig. 2B.7. Changes in the α/β ratio of the enzyme activity in both suspended matter (-O-) and macroaggregates (-O-) from the Dona Paula Bay during 1999-2000.

2B.3.6 Statistical Analyses

Bacterial production and enzyme activity in seawater and macroaggregates had no significant correlation with hydrography or nutrients (**Table 2B.3 &**

Chl *a* (r= 0.512, p<0.073) (**Table 2B.3**), whereas PBp had a significant linear correlation with Chl *a* (r= 0.625; p<0.01). However, TBp had no significant correlation with EPS (NaCl-soluble or EDTA-soluble) and TEP (**Table 2B.3**). Similarly, bacterial enzyme activity too did not have any correlation with any of the bulk particulate parameters. Bacterial production and enzyme activity in the macroaggregates had insignificant relationship with Chl *a*, EPS (NaCl-soluble and EDTA-soluble) and TEP (**Table 2B.4**). However, highly significant correlation was recorded between total bacterial production and enzyme activity in seawater samples only (**Tables 2B.3**).

The influence of seawater samples on the microbial activity in macroaggregates was also assessed. Bacterial production, abundance and extracellular enzyme activity in macroaggregate samples were correlated with suspended load, Chl *a*, EPS and TEP concentrations in the seawater samples (**Table 2B.5**). The EDTA-soluble EPS of the seawater samples had significant correlation with total α-glc activity (r= 0.639; p<0.001) and weak correlation with β-glc activity (r= 0.539; p< 0.06). Similarly, Chl *a* concentrations of water column had linear trends with bacterial production (r= 0.434) and total glc activity (**Table 2B.5**).

Table 2B.3: Pearsons correlation coefficients of bacterial production, enzyme activity and enzyme ratio with various particulate parameters of the seawater samples during 1999-2000 in the Dona Paula Bay.

Parameters	TBp	Total α-glcA	PA-α-glcA	Total β-glcA	PA-β-glcA	α/β ratio
TBA	-0.059	-0.310	-0.332	-0.371	-0.425	-0.215
TBp		0.677**	0.684**	0.643**	0.624**	0.519
Total α-glcA			0.996***	0.959***	0.865***	0.698**
PA-α-glcA				0.959***	0.889***	0.714**
Total β-glcA					0.954***	0.544*
Suspended load	0.208	-0.250	-0.172	-0.180	0.084	-0.047
Chl *a*	0.513	0.349	0.358	0.360	0.382	0.352
POC	0.003	-0.070	-0.070	0.014	0.096	-0.204
TEP	0.289	-0.018	-0.018	-0.010	-0.004	-0.179
NaCl-soluble EPS	0.152	0.102	0.090	0.305	0.361	-0.198
EDTA-soluble EPS	-0.167	0.220	0.259	0.223	0.306	0.329

*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

Table 2B.4: Pearsons correlation coefficient for bacterial production, enzyme activity and enzyme ratio with various particulate parameters of the macroaggregate samples during 1999-2000 in the Dona Paula Bay.

Parameters	TBp	Total α-glcA	PA-α-glcA	Total β-glcA	PA-β-glcA	α/β ratio
TBA	-0.136	0.370	0.384	0.408	0.445	-0.227
TBp		0.316	0.230	0.383	0.273	-0.190
Total α-glcA			0.994***	0.972***	0.963***	-0.078
PA-α-glcA				0.965***	0.972***	-0.067
Total β-glcA					0.988***	-0.213
Total macroaggregate	0.372	0.239	0.256	0.190	0.206	-0.10
Chl *a*	0.075	0.497	0.481	0.391	0.383	0.03
POC	-0.173	0.138	0.131	-0.008	-0.004	0.230
TEP	-0.076	-0.006	-0.001	-0.079	-0.080	0.349
NaCl-soluble EPS	-0.124	0.076	0.087	0.114	0.152	-0.230
EDTA-soluble EPS	-0.389	0.186	0.217	0.111	0.171	0.321

*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

Principal component analyses (PCA) of both seawater and macroaggregate samples were carried out to further assess the effect of various particulate parameters on the bacterial production and enzyme activity. The data were subjected to Varimax rotation and two factors were extracted from seawater data and three factors from macroaggregate data (**Tables 2B.6 & 2B.7**). The factor loadings of seawater samples and macroaggregates explained 84% and 71% of the data, respectively. When the factor loadings of seawater samples were plotted against each other (**Fig. 2B.8a**), TEP, NaCl-soluble

EPS and POC formed one group whereas TBp, enzyme activity, Chl *a* and EDTA-soluble EPS formed another group. Both suspended load and total bacterial count formed remained independent. Similarly, EPS (NaCl-soluble EPS and EDTA-soluble EPS), total macroaggregates and bacterial abundance in macroaggregates formed one group whereas enzyme activity, bacterial production and Chl *a* formed the other group (**Fig. 2B.8b**). TEP and POC remained independent of all the other parameters in macroaggregate samples.

Table 2B.5: Pearsons correlation of bacterial abundance, production and enzyme activity of macroaggregates with particulate parameters of suspended particulate matter.

Parameters	Suspended load	Chl *a*	POC	NaCl-soluble EPS	EDTA-soluble EPS	TEP
TBA	0.578*	-0.414	0.02	0.241	0.372	-0.422
TBp	-0.256	0.434	-0.318	-0.174	-0.168	0.197
Total α-glc	0.161	0.395	0.272	0.030	0.639**	-0.181
PA-α-glc	0.166	0.367	0.301	0.054	0.663**	-0.188
Total β-glc	0.042	0.307	0.202	0.073	0.539*	-0.256
PA-β-glc	0.058	0.263	0.213	0.083	0.580*	-0.260

*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

Table 2B.6. Factor loadings and eigen values obtained from various particulate parameters of the suspended matter after performing principal component analyses.

Parameters	Factor 1	Factor 2
Dry weight	-0.072	-0.949
Chl *a*	0.667	0.624
POC	0.810	0.544
EDTA-EPS	0.820	0.461
NaCl-EPS	0.696	0.614
TEP	0.545	0.618
Bacterial abundance	0.087	0.825
Bacterial production	0.853	0.480
Total-α-glc A	0.970	0.214
Total-β-glc A	0.937	0.146
Eigen values	7.642	1.616
Variance	5.711	3.547
Total	0.519	0.322

Table 2B.7. Factor loadings and eigen values obtained from various particulate parameters of the macroaggregates after performing principal component analyses.

Parameters	Factor 1	Factor 2	Factor 3
Dry weight	0.703	0.018	0.664
Chl *a*	0.050	0.590	-0.225
POC	0.077	-0.007	0.837
EDTA-EPS	0.796	0.011	-0.014
NaCl-EPS	0.764	-0.003	-0.046
TEP	-0.503	-0.094	0.605
Bacterial abundance	0.885	0.245	0.083
Bacterial production	-0.384	0.546	-0.264
Total-α-glc A	0.156	0.947	0.208
Total-β-glc A	0.161	0.941	0.097
Eigen values	3.407	2.512	2.094
Variance	2.954	2.497	1.690
Total	0.295	0.250	0.169

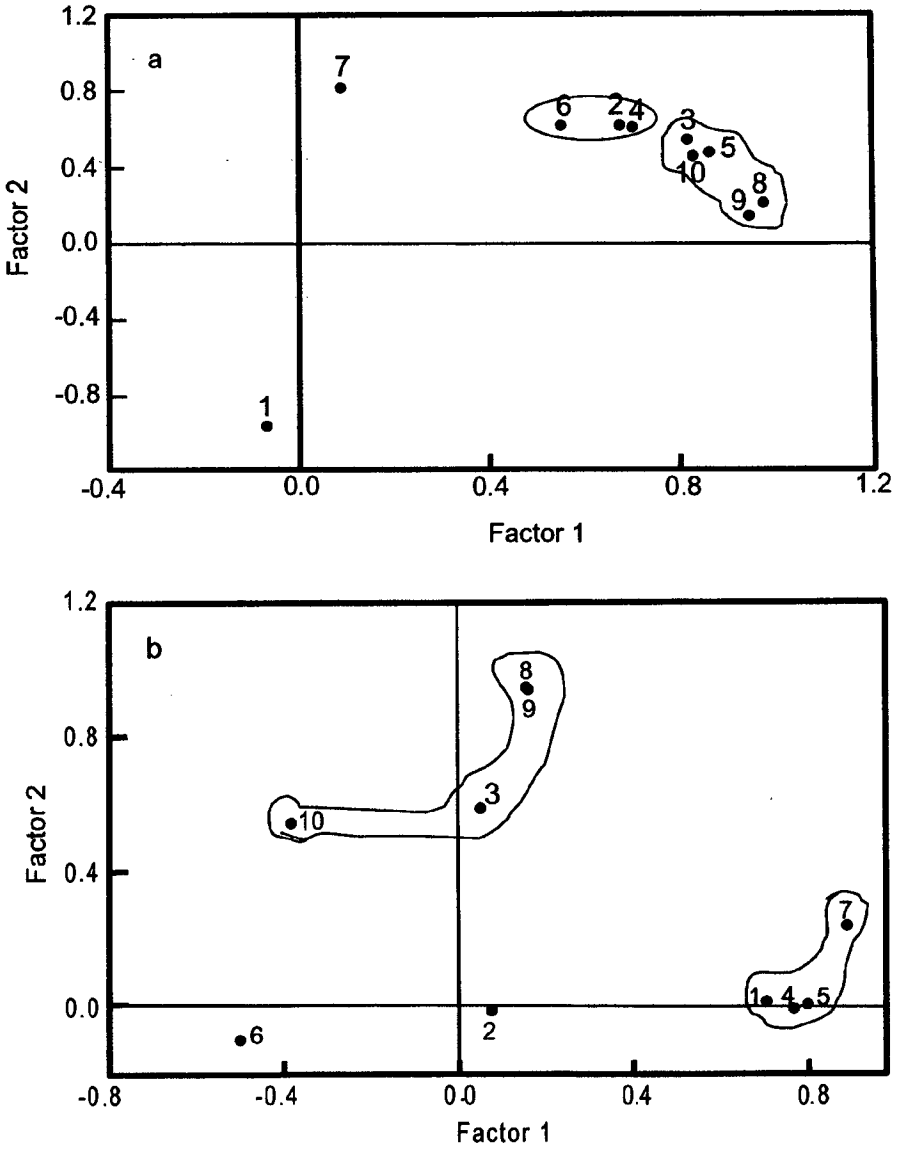

Fig. 2B.8 Varimax normalized PCA plots of various particulate parameters of seawater samples (a) and macroaggregate samples (b) collected from Dona Paula Bay. Each number represents a particulate parameter as shown below:

1: Suspended load or Total macroaggregates; 2: POC; 3: Chl *a*; 4: NaCl-soluble EPS; 5: EDTA-soluble EPS; 6: TEP; 7: TBA; 8: Total α-glc; 9: Total β-glc and 10: TBp.

2B.4 Discussion

2B.4.1 Ecto-glucosidase activity

The estimates of enzyme hydrolysis rates are influenced by the substrate concentration, incubation period and competitive inhibition of the substrate analogs by naturally occurring organic substrates (Hoppe 1983). In the present study, samples were inoculated with lower MUF-substrate concentrations and variable incubation time. Although 2 h incubation was optimum (**Fig. 2A.1a**), certain samples (especially macroaggregates) were incubated longer to ensure adequate hydrolysis. Lower concentration of MUF-substrate was preferred (**Fig. 2B.1b**) to minimise the interference from water column turbidity (Somville 1988).

Monsoon influenced seasonal variation in α- and β-glc activity was observed in both seawater samples and macroaggregate samples. The total α- and β-glc activity in the water column was in the range of 2.3 to 128 nM-C h^{-1} and 3 to 62.4 nM-C h^{-1}, respectively (**Table 2B.1**). Moreover, α- and β-glc activity showed similar seasonal behavior with exceptionally high activity during late monsoon (September '99) in the water column and macroaggregates (**Fig. 2B.4a - d**). There are not many studies on heterotrophic enzyme activity in tropical coastal waters (Rath et al 1993). Moreover, no information is available on the seasonal variations of bacterial heterotrophy in the coastal waters of India. Hence, comparison with other data from Indian waters was not possible. The range of heterotrophic enzyme activities at the study site were

similar to those reported for a mesotidal estuary (Cunha et al 2001; β-glc activity- 4.3 to 181.3 nM h^{-1}) but higher than those reported for a tropical coastal station (Rath et al 1993; α-glc- 0.3 to 1.6 nM h^{-1} and β-glc- 0.3 to 1.8 nM h^{-1}), eutrophic lake (Middleboe and Søndergaard 1993; β-glc- 7.8 to 22 nM h^{-1}) and temperate coastal waters (Nausch et al 1998; α-glc- 0.5 to 18 nM h^{-1} and β-glc- 0.8 to 12 nM h^{-1}). However, most of these works were carried out in temperate waters and hence direct comparisons are only subjective.

In seawater samples, extracellular enzyme activity did not show any correlation with other bulk particulate parameters like dry weight, POC and NaCl-soluble EPS **(Table 2B.4)**. On the other hand, Chl *a* and EDTA-soluble EPS had a linear trend with the enzyme activity. This is also reflected in the PCA plots (**Fig. 2B.8a**) as the enzyme activity is grouped with Chl *a* and EDTA-soluble EPS. On the other hand, enzyme activity in macroaggregates had positive trend with Chl *a* concentrations and no correlation with EPS and TEP (**Table 2B.5**), which is further substantiated in the PCA plots (**Fig. 2B.8b**) where the enzyme activity is grouped with Chl *a*. This suggests that although statistically significant correlations were not observed, Chl *a* and to some extent EDTA-soluble EPS appeared to influence microbial enzyme activity in both water column macroaggregate samples. The enzyme activity of macroaggregate samples showed moderate to significant correlation with EDTA-soluble EPS of seawater samples. From the correlations it appears that the composition of organic matter in the water column might also influence the macroaggregate associated bacterial enzyme activity. On the other hand,

factors like substrate quality, terrestrial inputs and river-runoff might also be important in regulating the bacterial activity in the study area.

The distribution of the two forms of glc enzymes have been widely used to assess the quality of the POM accumulating in the water column. It has been suggested that α-glc activities are higher when the particulate organic matter is freshly derived from phytoplankton and organic rich. On the other hand, higher β-glc activity highlights the accumulation of aged and decomposed aggregates in the water column (Decho 1995, Karen and Rassoulzadegan 1995, Decho and Herndl 1995). Hence the ratio of α-glc/β-glc was used to explain the quality of the particulate organic matter in the study area. The average α-glc/β-glc ratio of the seawater samples was not very different from macroaggregates (**Table 2B.1 & 2B.2**). However, the seasonal variations of the ratios indicate that the changes in the composition of macroaggregates were inverse to that of suspended particles during most of the sampling period (**Fig. 2B.8**). For example, phytoplankton blooms during September '99 (**Fig. 2A.6a; Chapter 2A**) appeared to influence the α–glc/β–glc ratios in the surface waters suggesting that blooms were one of the major sources of labile organic matter in the seawater samples (**Fig. 2B.8**). In contrast to the seawater samples, the ratios of the macroaggregate sample was lower during September '99. Similarly, the α/β-ratios of macroaggregates were 3-folds greater than that of seawater samples during March 2000 to April 2000 although no major blooms were observed during this period. One reason for such changes in particle composition could be a shift in the composition of

phytoplankton grazing community. Earlier reports from the study area have shown that the composition of the grazing community shifts from herbivores in post-monsoon (October to January) months to carnivores in pre-monsoon (February to May) (Gauns 2000). This might lead to increased contribution of detritus and fecal matter to the total macroaggregates during post-monsoon (October to January) and increased contribution of phytoplankton to the macroaggregates in the pre-monsoon months (February to May). Such a shift in grazing behavior changes the composition of the aggregates, which is also seen in the microbial activity.

2A.4.2 Bacterial production and abundance

Bacterial production is indicative of metabolically active cells and showed distinct seasonality during the study period ranging from 0.51 to 3.6 µg-C l^{-1} h^{-1} in the water column and 0.013 to 5.15 ng-C mg^{-1} aggregate h^{-1} (Table 1). The average bacterial production (0.39 to 3.6 µg-C l^{-1} h^{-1}) in the surface waters was higher than those previously reported in these waters (0.3 to 1.6 µg-C l^{-1} h^{-1}; Gomes et al 1991) but similar to those reported by Ram et al (2002) (0.6 to 6 µg-C l^{-1} h^{-1}) in these waters. The TBp values recorded in these waters were similar to those reported for a tidal temperate estuary (0.49 to 5.3 µg-C l^{-1} h^{-1}, Cunha et al 2000) but were much lower than those reported in near-shore tropical waters (9.2 to 15.5 µg-C l^{-1} h^{-1}; Rath et al 1993) and estuarine waters (0.5 to 10 µg-C l^{-1} h^{-1}; Roland and Cole 1999). Moreover, the mean bacterial abundance in the water column was 2.4 ± 2 x 10^6 cells ml^{-1}

which was similar to those reported by Ram et al (2002) (1.6 to 5 x 10^6 cells ml^{-1}) but higher than those reported by Devassy and Goes (1989) (0.203 to 0.405 x 10^3 cells ml^{-1}) and Gauns (2000) (0.043 to 0.36 x 10^5 ml^{-1}) in the Dona Paula Bay. However, these counts were lesser than those reported from the eastern coast of India (Ramaiah et al 1995) (10^7 to 10^8 cells ml^{-1}). The bacterial abundance at the study site was similar to those reported from a temperate estuary (2.8 to 3.5 x 10^6 cells ml^{-1}, Kirchman and Hoch 1988) but less than those reported from a mesotidal estuary (2.6 to 14 x 10^6 cells ml^{-1}, Cunha et al 2000).

In an estuarine environment, changes in the quality and quantity of organic matter and growth conditions can alter bacterial number, activity and community structure. During October to January, higher total bacterial production and abundance was recorded in both seawater samples (**Fig 2A.2a & 2A.3a**) and macroaggregates (**Fig. 2A.2b & 2A.3b**). These higher values coincided with phytoplankton blooms in the water column (**Fig. 2A.6a; Chapter 2A**). Similarly, higher Chl *a* concentrations in the month of June 2000 induced a similar bacterial response in TBp and total enzyme activity. The phytoplankton blooms during September-October '99 and June 2000 were dominated by diatoms like *Skeletonema*, *Nitzschia* and *Fragillaria* sp., which produce EPS during their growth. Bacteria actively degrade exudates of diatoms to meet their organic carbon requirement (Goto et al 2001). It is possible that release of these compounds might have increased the microbial

activity as evident from the high bacterial production and glucosidase activity (**Fig. 3a and 4a**).

Particle colonizing bacteria in aquatic environments form 5-10 % of total bacterial population in open oceans (Alldredge and Gotschalk 1990, Turley and Stutt 2000) and up to 90% of the total bacterial population in coastal and riverine ecosystems (Bell and Albright 1981, Crump and Baross 2000). In the present study, the percent of attached bacteria (3 to 79%) was similar to those reported earlier in these waters (4 to 98%, DeSouza 2000) but significantly higher than those reported for oligotrophic coastal waters (17%, Iriberri et al 1990), riverine waters (9.5%, Berger et al 1996) and in temperate lakes (11%, Friedrich et al, 1999). This was also reflected in the particle associated bacterial production (PA-Bp) that formed a major fraction (up to 75%) of the total bacterial production. Moreover, a major fraction of α- and β-glc enzyme activity (85% and 81 %, respectively) was also associated with particles and was significantly correlated with both the particle associated bacterial production and abundance (**Table 3 & 4**). This indicates that much of the enzymatic transformation of the organic matter in the water column was tightly coupled with production and bacterial abundance associated with the particles. It has been reported that bacteria growing on particles (seawater samples or macroaggregates) have higher growth efficiency, are bigger and hydrolyze and convert POM to DOM at a faster rate (Smith et al 1992, Decho and Herndl 1995, Grossart and Ploug 2000, Simon et al 2002). In contrast, bacterial production rates in macroaggregates have been found generally to

be same or less than in the surrounding waters (Kaltenbock and Herndl 1992, Muller-Niklas et al 1994, Simon et al 2002). In this study, the PABp was generally > the PFBp in both seawater samples and sinking macroaggregates. Thus, it appears that the organic matter broken down and released by particle-associated bacteria as dissolved organic matter (DOM) might not be sufficient to support the particle free bacteria. On the other hand, low PFBp might probably be due to less reactive DOM in the water column. In an earlier study, Wafar et al (1997) had suggested the importance of organic carbon derived by the dissolution of POM and its flux in these waters. The high particle associated bacterial production and heterotrophic activity recorded in this study might play an important role in sustaining the DOM levels in the study area.

The characteristics of macroaggregates are distinctly different from suspended particles. Although both contain organic matter from diverse sources and forms, macroaggregates are generally rich in detritus and fecal matter except during the course of a phytoplankton bloom wherein large number of intact phytoplankton cells is also found in the macroaggregates (Deuser et al 1981, Martin et al 1987). Sediment trap studies have shown that sinking particles are rich in organic carbon compared to suspended particulate matter (Falkowski et al 1994, Herndl 1995, Bhaskar et al 2000). Moreover, microbial activity (production, respiration and enzyme activity) in sinking aggregates like marine snow may exceed that of the surrounding waters (Karner and Herndl 1992, Grossart and Simon 1988, Unanue et al 1998). In

the present study, the specific bacterial production associated with macroaggregates were 28-folds greater than those in the seawater samples (**Table 2B.1 & 2B.2**). Similarly, specific enzyme activity was 7-folds higher in macroaggregate samples as compared to seawater samples (**Table 2A.1 & 2A.2**). Such higher cell-specific production and enzyme activity indicates the macroaggregate-associated bacteria actively mineralized labile form of organic matter from the macroaggregates. Moreover, it also points that the physiological state of the bacterial community associated with these particles were different from those in the surrounding waters. The sinking aggregates appeared to be hot spots of bacterial activity and remineralization compared to other forms of particulate matter.

2B.5 Conclusions

Temporal variations in bacterial abundance and production, enzyme activity and enzyme ratios were observed in both seawater samples and macroaggregates in the Dona Paula Bay. Seasonal changes in seawater samples and macroaggregate composition influenced the microbial activity at the study area. Most of the microbial activity was associated with particulate matter in both seawater and macroaggregate samples. Particle-associated bacteria therefore appeared to play a major role in organic matter transformations. Both phytoplankton biomass and EDTA-soluble EPS appeared to influence the bacterial enzyme activity in the seawater and macroaggregate samples. Although primary production is the major source of

organic carbon in these waters, inputs from POM including EPS and its turnover in the water column might be equally important in sustaining the microbial carbon demand.

CHAPTER 3

Isolation and characterization of EPS produced by *Marinobacter* sp.

Chapter 3

3.1 Introduction

Microorganisms (bacteria, phytoplankton and flagellates) produce high molecular weight, hydrated polymeric compounds called exopolysaccharides (EPS) during their life-cycle (Ducklow and Mitchell 1979). EPS may exist as capsules, sheaths, slimes (loosely attached to the cell wall), apical pads or mesh-like fibrils in the natural environment (Beveridge and Graham 1991, Costerton et al 1992, Herndl 1993, Hoagland et al 1993, Takeda et al 1998). Capsules are tightly bound to the cell wall by non-covalent linkages where as sheaths are linear EPS-containing structures surrounding chain of cells. Slime layer is a less organized form of capsule or sheath that diffuses into the surrounding environment (Wingender et al 1999). Microorganisms produce different forms of EPS to perform diverse functions.

Although not an essential feature, EPS does provide structural and functional stability to microbial assemblages in the natural environment (Decho 2000). Most microorganisms produce EPS either for attachment to substratum (adhesion), formation of micro-consortium/biofilms or binding to other particulate matter (cohesion or aggregation). EPS produced for attachment by microorganisms may influence biofouling by conditioning the substratum (Characklis and Escher 1988). Other functions like gliding motility, protection against osmotic shock, predation, desiccation and detoxification of toxic compounds, nutrient sequestering, chelation of metals, horizontal transfer of

genetic material etc. have also been attributed to microbial EPS (Decho 1990, Hoagland et al 1993).

EPS in the natural environment is generally heteropolymeric (made of different monomeric units) (Decho 1990, Wingender et al 1999). EPS contain non-sugar components like uronic acid, methyl esters, sulphates, pyruvates, proteins, nucleic acids and lipids (Corpe 1980, Sutherland 1980, Cadieux et al 1983, Hoagland et al 1993, Wingender et al 1999, Sutherland 2001). EPS also contain divalent metal cations that act as ionic bridges linking adjacent polysaccharide chains (Rees 1969 & 1972, Fletcher 1980). The presence of side-linkages and organic molecules influence the overall charge, stability, binding capacity, rheology and solubility of the polymer (Hoagland et al 1993).

Many bacterial cultures produce different types of EPS during its life-cycle. For example, most bacteria produce capsular form of EPS during the exponential growth phase and slime type EPS during the stationary growth phase (Decho 1990). Similarly, the chemical characteristics of EPS changes with the age of the culture (Gloaguen et al 1995), nutrient levels (Wiliams and Wimpenny 1978, De Philippis et al 1991) and growth conditions (Smith and Underwood 1998, Jahn et al 1999).

In the marine environment, the bacterial numbers are in the range of 10^5 to 10^8 ml^{-1} (Cho 1991, Ramaiah et al 1998, Bode et al 2001). Moreover, up to 45% of organic carbon like glucose incorporated by bacteria is converted into EPS, which is then released into the surrounding waters (Stoderegger and

Herndl 1998). Heissenberger and Herndl (1992) have shown that metabolically active bacteria produce EPS throughout their living period and may contribute to the EPS concentrations in marine waters, especially in oligotrophic growth conditions. Based upon the information available on the ubiquitous distribution of bacterial EPS in the oceans and its contribution to the organic carbon pool (Stoderegger and Herndl 1998), its clear that bacterial EPS might play an important role in regulating various marine processes and fuelling the marine food-web (Decho 1990). However, most of the laboratory and *in situ* studies on the factors regulating important marine processes like aggregation, flux and trophic web have focused on the role of phytoplankton and its extracellular polymers (Decho 1990, Sell and Overbeck 1992, Passow et al 1994, Niven et al 1995, Passow and Alldredge 1995, Passow et al 2000, Prieto et al 2001). Thus, very little is known about the behaviour of bacterial EPS in the marine environment despite its wide distribution and high abundance.

A seasonal variation in the distribution of EPS as discreet particles (TEP) or in association with other particulate matter has been observed in near-shore waters (see Chapter 2a). Some forms of EPS showed good correlation with bacterial production suggesting its utility as a possible organic carbon source. On the other hand, EPS in the form of TEP was inert to bacterial activity and appeared to be a major sink of organic carbon in these waters (Chapter 2b). Keeping in mind the ecological significance of the EPS in general and the limited information available on the role of bacterial EPS in marine processes, a study was carried out to characterize the bulk and molecular chemistry of

EPS isolated from a marine bacterium. Furthermore, experiments were also carried out to assess the effect of bacterial growth conditions on EPS production.

3.2 Material and Methods

3.2.1 Isolation and screening of bacterial cultures for EPS production

Surface seawater samples from Dona Paula Bay were collected in 500 ml sterile flasks. Similarly, sediment samples were collected from Dona Paula and mangrove forests along the Zuari river. Macroalgal samples were collected from the intertidal regions of Dona Paula Bay. In order to isolate bacterial cultures, sediments and macroalgal samples were suspended in sterile filtered seawater, vortex mixed, serially diluted and appropriate dilutions were plated on Zobell Marine Agar (ZMA) plates. Similarly, seawater samples were serially diluted and plated on ZMA plates. The plates were incubated at room temperature (30 ± 2°C) for 48 h. Bacterial colonies with mucoidal appearance or colonies that had a sticky surface were picked up and purified by repeated streaking on ZMA. On an average 5 to 7 colonies were randomly selected from each plate during every sampling for further purification. The purified cultures were then transferred to ZMA slants and stored at 4°C. Bacterial cultures obtained from the Materials & Material Research Division (MCMRD) culture collection were also screened for their potential to produce EPS.

Loopful of cultures from the slants were transferred into test-tubes having 5 ml of BSS medium (Appendix Ia) supplemented with 1% glucose as the sole source of carbon and grown at room temperature (28 ± 2°C) for 24-48 h. The cultures were sub-cultured twice in the same medium before measuring their EPS production. Cells were centrifuged at 10,000 rpm (16750 g) for 30 mins, supernatant was filtered through 0.22µm filters and dialysed against glass-distilled water at 4°C using 8000 daltons (8 kDa) MW cut-off dialysis bags (Decho 1990). After dialysis, the dialysate was made to a known volume and analysed for total carbohydrates using the phenol-sulphuric acid method (Dubois et al 1956). Bacterial isolate that produced the highest amount of EPS was chosen for further studies.

3.2.2 Identification of the selected culture

The selected EPS producing bacterium was identified using both conventional physiological and biochemical tests followed by the molecular identification method based on 16S-rRNA sequencing.

a) *Conventional identification methods*- The bacterial isolate was studied for morphological, 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, the culture could be identified up to its family. For further identification, molecular methods were used.

b) *Molecular identification method*- The 16S-rRNA sequencing procedure can be divided into 3 stages: i) chromosomal DNA extraction, ii) gene amplification by polymerase chain reaction (PCR) and iii) DNA sequencing and analyses.

i) *Chromosomal DNA extraction*

The chromosomal DNA was extracted by repeated freezing (-80°C) and heating (at 95°C) of the bacterial cells for 30 min each in Tris buffer at pH 8. The cells were centrifuged at 10,000 x g for 10 min at 4°C to separate the cells. To the supernatant, few microlitres of proteinase K, DNAase free RNAase and SDS were added and incubated at 37°C for half an hour. Further extraction and precipitation was done following Sambrook et al (1989).

ii) *Gene amplification*

An approximately 1500 bp-segment of the 16S-rRNA gene was amplified by PCR with a pair of primers targeting the 5' end (sequence-AGAGTTTGATC(AC)TGGC) and the 3' end (sequence-TACCTTGTTACGACTT; MWG, Germany), respectively. The PCR cycle involved denaturing of the strand at 95°C for 5 min followed by 30 cycles of annealing (for 1 min at 40°C), extension (for 3 min at 72°C) and denaturing at 95°C (for 1 min). A final extension for 10 min at 72°C was carried out before the amplified sequences were loaded on 0.8% agarose gel and separated by gel electrophoresis. The gel was then immersed in ethidium bromide for 2 h

and the products were detected using a transilluminator. The PCR products were then purified using QIAquick Purification Kit (Qiagen, USA).

iii) *DNA sequencing and analyses*

The DNA sequencing was carried out using DYEnamic Direct cycle sequencing kit US79535 (Amersham Life Science Inc.) and a Li-Cor Long 4200 automated DNA sequencer (MWG, Germany). Sequencing primers used were GM 3F (5'-AGAGTTTGATC(AC)TGGC-3') and GM 8R (5'-TGGGTATCTAATCCT-3', MWG, Germany) and were labelled with IRDyeTM800. The sequencing protocol consist of an initial denaturing step at 95°C for 5 min, followed by 30 cycles of denaturing 95°C for 0.5 min, annealing at 40°C for 0.5 min and 1 min extension at 70°C. The reaction was immediately cooled to 4°C and maintained till loading in the sequence gel. Electrophoresis was carried out at 45°C for 16h (Biocym, Germany). The sequences thus obtained were aligned and compared with those available in the Genebank of the National Centre for Biotechnology Information (NCBI), USA (Altschul et al 1990).

3.2.3 Optimization of growth conditions of Marinobacter sp.

In order to optimize growth conditions, *Marinobacter* culture (1% v/v) was first grown in test-tubes having 5 ml BSS medium (Appendix-Ia) supplemented with 1% glucose and low MW fractions of yeast extract (0.05%) and peptone (0.1%) at 28 ± 2°C (pH 7.6) for 48 h. Before use, 100 ml 10x stock solutions of

yeast extract and peptone were passed through 10 kDa MW cut-off membrane filters using Amicon filtration system (Amicon, USA) to remove high molecular weight contaminants.

1) *Effect of glucose concentration- Marinobacter* sp. was grown in BSS medium containing varying glucose concentrations (0%, 0.5%, 1%, 2%, 3% and 5%) to study the effect of glucose concentration on the growth and EPS production.

2) *Effect of nitrogen source and concentration-* The effect of nitrogen source was assessed by growing the culture in glucose optimized BSS media supplemented with 0.1% of different nitrogen sources like NH_4Cl, $NaNO_3$, urea, $(NH_4)_2SO_4$ and KNO_3. The nitrogen source that enhanced EPS production was selected. The concentration of this N-source was then varied to assess the influence of increasing nitrogen concentration (0%, 0.0026%, 0.0052%, 0.013% and 0.026%) on EPS production.

3) *Effect of phosphate concentration-* In order to assess the effect of phosphate concentrations on the growth and EPS production of *Marinobacter,* the culture was grown in BSS medium (optimized for glucose and nitrogen) having increasing phosphate concentrations (0%, 0.017%, 0.034%, 0.056% and 0.085%).

4) *Effect of magnesium concentration-* The culture was grown in the presence of increasing magnesium concentrations (0%, 0.0048%,

0.0096%, 0.0168% and 0.024%) to assess the role of magnesium concentrations in EPS production and bacterial growth.

During each experiment, the bacterial growth was monitored by measuring the turbidity at 540 nm. The amount of EPS produced by the culture was estimated by the phenol-sulphuric acid method (Dubois et al 1956).

3.2.4 Growth and EPS production of Marinobacter sp.

The bacterial culture was grown in 1 l capacity conical flask containing 500 ml optimized BSS medium (Appendix Ib) at pH 7.6. The culture was grown for 170 h during which sub-samples (5 ml) were withdrawn at regular intervals for both EPS production and growth. Bacterial growth was estimated by turbidity measurements at 540nm. In order to extract EPS, the cells were first centrifuged at 10,000 rpm (16750 g) for 30 mins and the supernatant (dissolved fraction) was decanted. The dissolved fraction was filtered through 0.22 µm filters. The cell pellet was resuspended in 10mM EDTA solution, vortexed for 15 mins and centrifuged at 10,000 rpm (16750 g) for 10 mins. The supernatant thus obtained was mixed with the dissolved fraction and then dialysed against distilled water using 8kDa MW cut-off dialysis bag for 48 h. During the dialysis period, water was changed every 10 h. After the dialysis, 1 ml of the dialysate was used to estimate EPS as carbohydrates following Dubois et al (1956).

3.2.5 Bulk EPS production for chemical characterization

Pure culture of *Marinobacter* sp. was grown in 2 l flasks containing 1 l of the optimized BSS medium adjusted to pH 7.6. The culture was grown at 28°C ± 2°C over a period of 14 days. At the end of the incubation period, the cells were harvested by centrifugation at 10,000 rpm (16750 g) for 30 mins and the supernatant was collected. The cell pellet was treated with 10mM EDTA as described earlier to extract cell-bound EPS. The EDTA-extracted EPS was pooled with the supernatant EPS solution and filtered through 0.22 µm filters, concentrated at 35°C to less than 50 ml using a rotary evaporator.

The concentrated EPS was then precipitated using ice-cold absolute ethanol (Bhosle et al 1995) while continuously stirring and kept at 4°C overnight. The supernatant was decanted; the precipitate was redissolved in minimum volume of distilled water and dialysed for 48 h using 8 kDa MW cut-off dialysis bags against distilled water to remove salts. During dialysis, the water was changed every 10 h. The high molecular weight EPS retained in the dialysis bag was then reprecipitated, lyophilised and stored at –20°C until further analyses.

3.2.6 Chemical characterization of EPS

The EPS of *Marinobacter* sp. was analysed for its bulk chemical characteristics like total carbohydrates (TCHO), proteins, uronic acid (UA),

sulphates, total carbon and nitrogen. The EPS sample was also analysed for its monosaccharide and amino acid constituents.

3.2.6a Reagent preparation

Protein assay

Bicinchoninic acid (BCA)- BCA reagent for protein analyses was freshly prepared by adding 1 ml of 4% $CuSO_4$ to 50 ml of BCA solution (Sigma Chemicals USA). The reagent was prepared for immediate use.

Uronic acid assay

Sulfamic acid- Four molar sulfamic acid solution was prepared by adding sulfamic acid in half volume of deionized water under continuous mixing and dissolved by drop-wise addition of saturated KOH. Once completely dissolved, the final volume was made with distilled water. This reagent was used to reduce the caramelization of sugars during uronic acid estimation.

Metahydroxy biphenyl reagent- Biphenyl reagent for uronic acid estimation was prepared by dissolving 150 mg of metahydroxy biphenyl in 100 ml of 0.5% (w/v) NaOH solution. This solution is stored under refrigeration.

Borax reagent- The borax reagent is prepared by adding 2.86 g of sodium tetraborate in 100 ml of concentrated sulfuric acid.

Sulphate assay

$BaCl_2$-gelatin reagent- The $BaCl_2$-gelatin reagent for sulphate estimation was prepared by dissolving 0.5% gelatin in warm water (60-70°C). The solution was stored overnight at 4°C. The following day, 0.5% of $BaCl_2$ was added and the resultant cloudy solution was allowed to stabilize for 2-3 h at room temperature before using for sulphate estimation.

3.2.6b Analytical methods

1. Bulk characterization

Carbohydrates (TCHO)- TCHO concentrations were estimated following the phenol-sulphuric method (Dubois et al 1956) with a slight modification. A sub-sample of the lyophilised EPS was weighed and dissolved in an appropriate volume of deionized water. If the samples were in the form of clear solution, EPS sample was directly used for total carbohydrate estimation. One ml of the EPS solution was mixed with 1 ml of cold 5% phenol and 5 ml of concentrated sulfuric acid was added to develop colour. The intensity of the colour was measured spectrophotometrically at 490 nm. Glucose was used as a standard for calibration of the method.

Proteins- The protein content of the EPS was estimated following Smith et al. (1993). A known amount of the EPS was weighed, dissolved in appropriate volume of distilled water and used directly for protein estimation. In order to analyze the protein content of the EPS, 100 µl of the EPS sample was

transferred into clean test-tubes and 2 ml of bicinchoninic acid (BCA) reagent was added to it. The samples were then incubated at 37°C for 30 min to complete the reaction and develop color. The intensity of the color formed was measured spectrophotometrically at 562 nm. Bovine serum albumin was used as a standard for the calibration of the method.

Uronic acid (UA)- UA content in the EPS was measured following the modified meta-hydroxy biphenyl method of Filisetti Cortiza and Carpita (1993). The modified method remains the same as the old meta-hydroxy biphenyl method (Blumenkrantz and Asboe-Hansen 1973) except for the pretreatment with sulfamic acid. A known amount of EPS was dissolved in deionised water and 0.4 ml was taken in a clean test-tube. To this, 40 µl of 4 M sulfamic acid was added and mixed thoroughly. After mixing properly, 2.4 ml of sulfuric acid-tetraborate solution was added and vortex mixed. The test-tube was then placed in boiling water bath (100°C) for 20 min and then immediately placed in crushed ice. Once cooled, 80 µl of metahydroxy biphenyl was added to the mixture, vortex mixed and the colour developed was measured at 525 nm. Galacturonic acid was used as a standard for the calibration of the method.

Sulphate estimation- Sulphates in the EPS were estimated following $BaCl_2$-gelatin method (Dodgson and Price, 1963). A known amount of EPS was dissolved in deionized water and 1-2 ml of the sample was transferred into glass ampoule and dried under flow of nitrogen gas. To this ampoule, 1 ml of 1 N HCl was added and the sample was flushed with nitrogen. Thereafter, the ampoule was sealed and the sample was hydrolysed at 105°C for 17 h. After

hydrolyses, the sample was cooled and the content was transferred to test-tube containing 3.8 ml of 3% trichloro acetic acid (TCA) solution. To this mixture, 1 ml of $BaCl_2$-gelatin reagent was added and mixed thoroughly. The solution was kept at room temperature for 15-20 min and the turbidity formed was measured at 360 nm. Potassium sulphate dissolved in 1 N HCl was used as a standard for calibration.

In order to estimate blanks in all the above estimations, deionized water was used in place of the samples and analysed.

Carbon and Nitrogen- Total carbon and nitrogen content of the bacterial EPS was analysed using a ThermoFinnigen CNS analyzer. A known amount of dry EPS sample was weighed accurately in tin foils and combusted in the CNS analyzer. Methionine was used as a standard for the calibration of the method. Empty tin cups were used as blanks.

FTIR analyses- Fourier Transformed Infra-Red absorption spectrum of the EPS sample was obtained following KBr technique. Small amount of lyophilised EPS sample was mixed with KBr crystals using mortar and pestle, placed in cup and compressed to form a thin pellet. The spectrum of the pellet was then obtained using a Shimadzu FTIR spectrophotometer.

2. Molecular characterization

Monosaccharide composition- Freeze-dried EPS sample was weighed and dissolved in a defined volume of deionised water. One ml of the EPS was

transferred into 10-ml ampoule and mixed with 10 µl of 10M HCl (final concentration 100 mM HCl), flushed with nitrogen, sealed and hydrolysed at 100°C for 20 h. After hydrolysis, the samples were dried at 60°C over a continuous flow of nitrogen gas. The dried samples were suspended in 10 ml of deionized water and passed through ion-exchange Dowex 50 W-X 8 columns (H^+ form, 100 mesh, Sigma USA). Aldoses were then detected amperiometrically with a HPLC system equipped with a Carbopac PA 10 column (Dionex, USA) using 20 mM NaOH as an eluent (HPLC-PAD; Borch and Kirchman 1997). The solvents (MilliQ water and 200 mM NaOH) were pumped at the rate of 1 ml/min in a ratio of 0.1 ml NaOH and 0.9 ml MilliQ water. A standard sugar solution containing 100mM concentration of glucose, galactose, rhamnose, mannose/xylose, fucose, fructose and arabinose (Sigma Chemicals, USA) was prepared and used for the calibration of the method.

Amino acid composition- The total hydrolysable amino acid (THAA) composition and concentrations in the EPS were analysed by HPLC with a fluorescence detector (Lindroth and Mopper 1979, Bhosle and Wagh 1997). Suitable amount of EPS was weighed and dissolved in an appropriate amount of deionised water. Equal volumes of EPS sample and 12N HCl, 20 µl of ascorbic acid and 20 µl of 100 µM α-aminobutyric acid (internal standard) were taken in clean glass ampoules, flushed with nitrogen, sealed and hydrolysed at 110°C for 20h. After hydrolysis, the samples were dried completely over a stream of nitrogen at 60°C and reconstituted in 1ml of deionised water.

Fluorescent amino acid derivatives were prepared by mixing 50 µl of the hydrolysate with 50 µl of o-phthaldialdehyde (OPA) and 2-mercaptoethanol at pH 9.5 for 2 min. The samples were then separated using reverse-phase HPLC using a binary solvent system i.e. methanol and sodium acetate buffer (40 mM sodium acetate having 3% tetrahydrofuran (THF) at pH 6.0). The retention time of the standards were matched with those of the samples to identify the different amino acids and the concentrations were calculated by integrating the peaks relative to those of standard component peaks. The method was calibrated by running standards having all the 16 amino acids with a concentration of 20 nM each.

For blanks, deionised water instead of samples was used, processed and analysed as above.

3.3 Results

3.3.1 Screening and identification of EPS producing bacterial isolate

About 150 bacterial isolates obtained from various natural samples and departmental culture collection were screened for EPS production. The morphology and the gram characteristics of some of the isolates and their EPS production estimated after 48 h of incubation in 1% glucose supplemented BSS medium is shown in **Table 3.1.** From the table, its clear that isolates P3R and Ti-21d(3) produced the maximum amount of EPS. Based upon its ability for EPS production, a bacterial isolate (P3R) obtained from the sediments of mangrove forest along the Zuari river was chosen for

Table 3.1: Some of the bacterial isolates examined for their EPS production after 48 h of incubation at 28 ± 2°C. The cultures were grown in 1% glucose supplemented BSS medium.

Bacterial isolates	Growth in 1% glucose medium in 48 h	EPS production (μg ml^{-1})	Cell-morphology and Gram characteristics isolated on ZMA plates
Seawater	—		
SW 1	+ +	9.5	Gram negative cocco-bacilli
SW 2	+	3.6	Gram negative short thick rods
SW 4	+	2.6	Gram negative long rods
SW 6	+	8.9	Gram negative, short thick rods
SW 6b	+ +	12.6	Gram negative coccobacilli
SW 7	+ +	9.1	Gram negative coccobacilli
SW 10	⊥	3.3	Gram negative short rods
SW 12	⊥	2.1	Gram negative long slender rods
SW 14	+ ⊥	1.7	Gram negative coccobacilli
SW 15	+ ⊥	2.6	Gram negative short rods
SW 17	+ ⊥	2.6	Gram negative coccobacilli
SW 21	+ ⊥	0.9	Gram negative coccobacilli
Sediment			
Sed. I	+ +	2.6	Gram negative short fat rods
Sed. II	+	5.2	Gram negative medium sized curved bacteria
Sed. II(a)	+ +	2.9	Gram negative short rods
P3R	+ + +	20.2	Gram negative short medium sized rods
Macroalgae			
Algal(I)	-	-	Gram negative medium sized thin rods
Departmental collection			
Ti-21d(3)	+ + +	20.2	Gram negative short rods
Ti-21d(5)	+ +	13.5	Gram negative medium sized rods
Ti-21d(16)	+ +	11.0	Gram negative short thick rods

- = no growth, ⊥= poor; + = moderate; + + = good; + + + = excellent

further studies. The cells were gram-negative short-rods, non-sporulating and motile that produced creamy white, circular colonies with smooth shiny surface when grown on Zobell-Marine agar. Other tests carried out indicated that the organism was facultative aerobic, both catalase and oxidase positive and reduced nitrate (**Table 3.2**). The culture was found to utilize many sugars as sole carbon source and was non-fermentative (**Table 3.3**). Based upon these characteristics of the isolate, the culture was identified as a member of

Table 3.2: Typical culture characteristics of *Marinobacter* sp.

Examination	Observation
Gram characteristics	Gram negative
Cell morphology	Short rods
Spores	Non-sporulating
Motility	Motile
Pigmentation	Creamy white
Colony shape	Small round
Elevation	Low convex
Consistency	Butyreous
Oxidase	Positive
Catalase	Positive
Indole production	Negative
Citrate	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Nitrate reduction	Positive
Urea	Positive
Hugh-Liefson's medium	Facultative aerobic
TCBS agar	No growth
MaConkey agar	No growth

the family *Alteromonadeacae* following Bergey's Manual of Systematic Bacteriology (1984). The culture was identified as *Marinobacter* sp. following the 16S-rRNA base sequences (**Fig. 3.1**) and showed 99% similarity with a strain *Marinobacter sp. Trimyema*-2.

Table 3.3: Fermentative properties of *Marinobacter sp.*

Fermentation Tests	Acid	Gas	Alkaline
Glucose	+	-	-
Sucrose	+	-	-
Galactose	+	-	-
Lactose	+	-	-
Maltose	+	-	-
Xylose	+	-	-
Arabinose	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
Mannose	+	-	-
Starch	+	-	-
Inositol	-	-	-
TSI slant	Butt	-	Slant

GTTTgATCATGRaTCAGATTGAACGCTGRCGRcAgGCTTAAcAcATgCAAgTCGAGCGGT
aACAGGGGGAGCTTGCTCCCTGcTGACGAGCGGCGGACGGGTGAGTAATGCATAGG/
ATCTGCCCAGTAGTGGGGGATAGCCCGGGGAAACCCGGATTAATACCGCATAACGCc
TACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTATTGGATGAGCCTATGTCGG/
TTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAGA
GGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAgCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAACCTGATGGTTAATACCC
ATTGGGCTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG(
CGGTTTGGTAAGCGAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTCGAA
CTGCCAGGCTAGAGTATGGTAGAGGGTAGTGGAATTTCCTGTGTAGCGGTGAAATGC
GTAGATATAGGAAGGAACACCAGTGGCGAAGGCGGCTACCTGGACCAATACTGACGC
TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG1
AAACGATGTCAACTAGCCGTTGGGACTCTTGAAGTCTTAGTGGCGCA-
CTAACGCACTAAgTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA/
TTgACGGGGGCCCGCACAAGCGTGGAGcaTGTGGTTTAATTCC.

Fig 3.1. 16S-rRNA sequence of *Marinobacter sp.* obtained after amplifying 1500 bp-segment of the 16S rRNA gene with a pair of primers targeting at the leading (5'-AGAGTTTGATC(AC)TGGC-3') and terminating end of the 16S rRNA gene (5'-TAC CTT GTT ACG ACT T-3'). Sequencing primers used were GM 3F (5'-AGAGTTTGATC(AC)TGGC-3') and GM 8R (5'-TGGGTATCTAATCCT-3'), both labeled with IRDveTM800.

3.3.2 Optimization of growth medium for EPS production

1) Effect of glucose concentration- Marinobacter sp. showed maximum growth when the medium was supplemented with 3% glucose (**Fig. 3.2**). However, EPS concentration was maximal when the culture was grown in 1% glucose. Thereafter, EPS concentrations did not increase with the increase in glucose concentrations, although cell growth increased up to 3% glucose (**Fig. 3.2**).

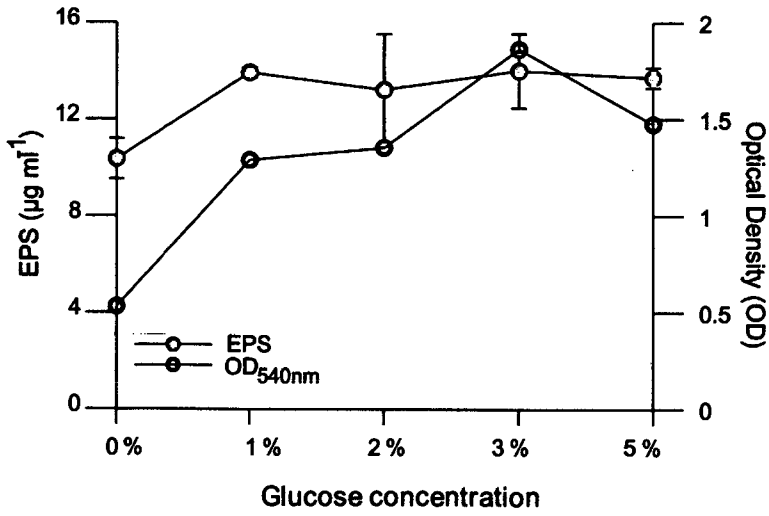

Fig. 3.2 Effect of glucose concentrations on the growth and EPS production of *Marinobacter* sp.

2) Effect of different nitrogen sources and concentrations- Ammonium chloride was most suitable source of nitrogen amongst the five nitrogen sources examined for both bacterial growth and EPS production (**Fig. 3.3a**). Thus, ammonium chloride was used to assess the effect of nitrogen concentrations on EPS production. Its clear from the graph (**Fig 3.3b**) that there was an almost 1.5-fold increase in EPS production by the culture when the medium was supplemented with 0.0026% N. Thereafter, the EPS concentration decreased with increasing N-concentration whereas cell density showed a small initial increase followed by a decrease in cell density with increase in N-concentrations.

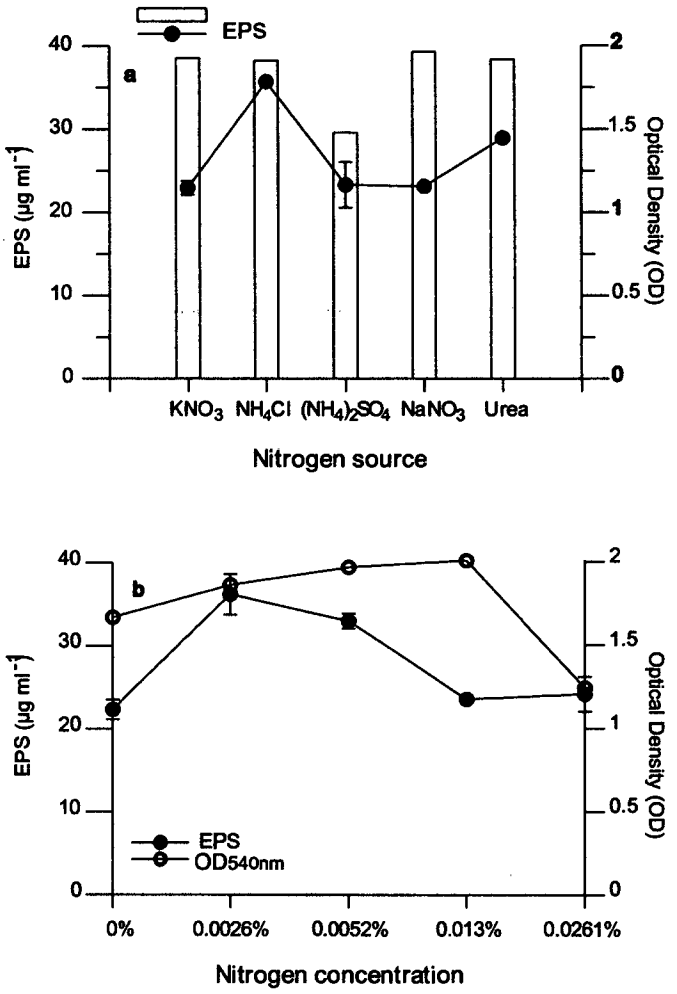

Fig. 3.3 Effect of various nitrogen sources (a) and different nitrogen concentrations (b) on the growth and production of EPS by *Marinobacter* sp.

3) Effect of phosphate concentration- EPS production by the culture was maximal when the medium was supplemented with 0.017% phosphate (**Fig. 3.4**). On the other hand, cell density increased with increase in phosphate concentrations followed by a decrease when the medium was supplemented with 0.085% phosphate.

4) Effect of magnesium concentration- Increasing concentrations of magnesium appeared to support EPS production more than cell growth. In contrast, there was not much difference in the cell density except for a sharp increase in the medium supplemented with 0.024% magnesium concentrations (**Fig. 3.5**).

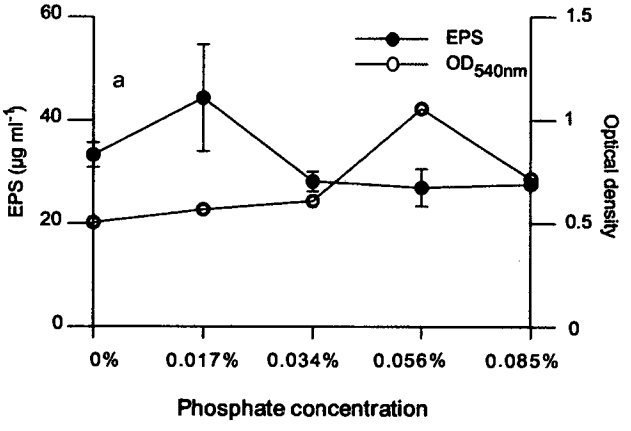

Fig. 3.4 Effect of different phosphate concentrations on the growth and EPS production by *Marinobacter* sp.

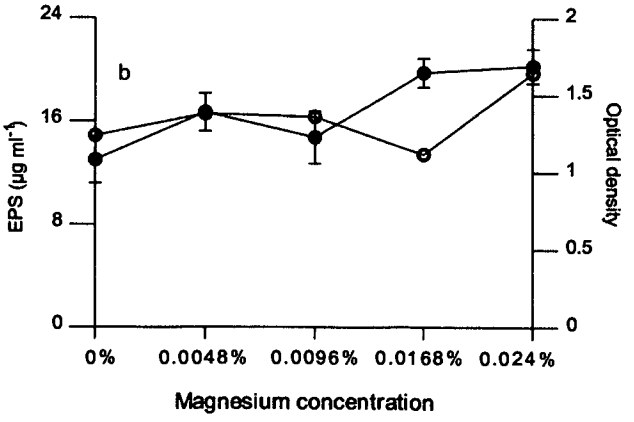

Fig. 3.5 Effect of different magnesium concentrations on the growth and EPS production by *Marinobacter* sp.

3.3.3 Growth and EPS production

The growth curve of the bacterium is shown in **Figure 3.6**. The culture growth increased gradually from an optical density of 0.04 at 0 h to 0.819 after 120 h. Thereafter, the growth of the culture did not vary much until the end of the incubation. The production of EPS was however different. In the beginning, EPS concentration rose from 9 μg ml^{-1} at 6 h to 18.8 μg ml^{-1} after 20 h of incubation and did not vary much until 61 h. Thereafter, the EPS concentration increased to 26.9 μg ml^{-1} between 61 h and 100 h followed by a decrease at the end of the experiment.

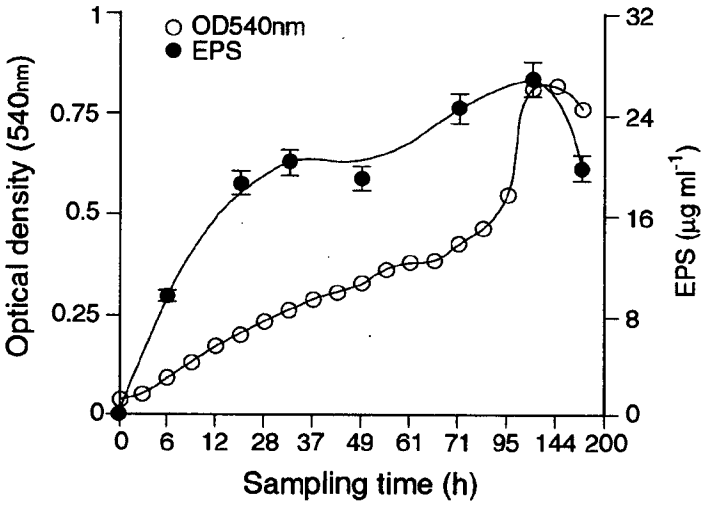

Fig. 3.6 Growth curve and EPS production of *Marinobacter* sp. grown in BSS medium supplemented with 3% glucose, 0.0026% N, 0.05% yeast extract and 0.25% peptone.

3.3.4 Chemical characterization

Bulk characterization

The chemical composition of the *Marinobacter* EPS is shown in **Table 3.4**. The EPS was enriched in proteins (438.8 μg mg^{-1}) relative to carbohydrates (168.9 μg mg^{-1}). The polymer contained 175.8 μg mg^{-1} uronic acid and 26.9 μg mg^{-1} sulphate. The C:N ratio of the polymer was 2.81.

Table 3.4: Bulk chemical composition of EPS isolated from *Marinobacter* sp. in comparison with EPS composition of other bacterial isolates.

Parameters	TCHO (μg/mg).	Proteins (μg/mg)	Uronic acid (μg/mg)	Sulfates (μg/mg)	C:N (w:w)	Reference
Marinobacter sp	168.9	438.9	175.8	26.9	2.81	Present study
Vibrio sp.	510.9	17.93	148.89	--	--	Majumdar et al (1999)
Halomonas eurihalina	363	102	12	--	--	Bejar et al (1998)
A. infernus	570	40	420	88	--	Guezennec et al (1998)
A. macleodii	420	40	380	50	--	Rougauex et al (1996)

FTIR spectra

The FTIR-spectra of bacterial EPS showed bands of carbohydrates at 1029.9 and 1107.1 cm^{-1}. On the other hand, carboxylate and sulphate peaks

were observed at 1612.4 cm^{-1} and 613.3 cm^{-1}, respectively in the EPS of *Marinobacter* (**Fig. 3.7**).

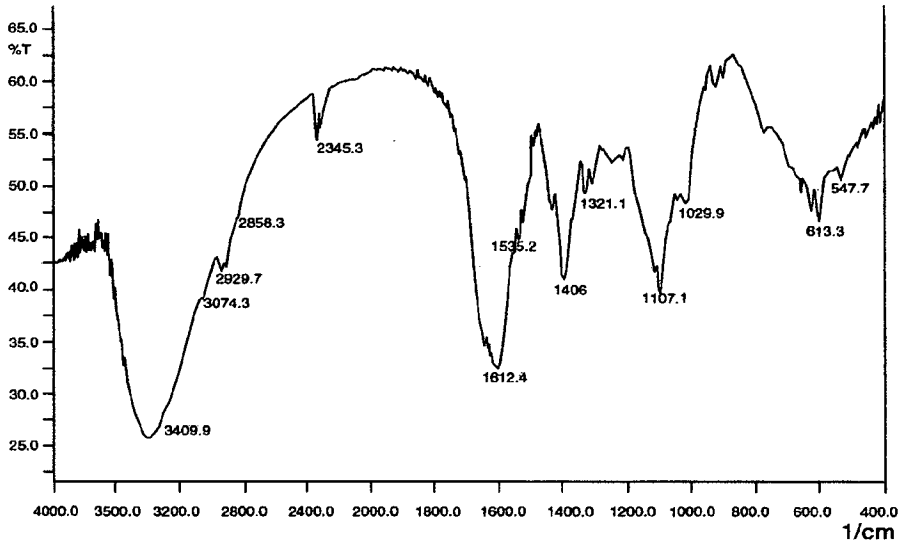

Fig. 3.7. FTIR-spectrum of bacterial EPS

Monosaccharide concentrations

Glucose was the most abundant (79.9%) monosaccharides in the bacterial EPS followed by galactose (17.6%) (**Table 3.5**). Mannose/xylose and fructose were present in smaller amounts (< 3%). Pentoses like ribose and arabinose and deoxy sugars like rhamnose and fucose were not detected in the EPS (**Fig. 3.8**).

Monosaccharides	Mole%
Fuc	---
Rib	---
Rha	---
Ara	---
Gal	17.6
Glu	79.8
Man+Xyl	1.5
Fru	1.1

Table 3.5: Monosaccharide (Aldose) composition (mole%) of EPS isolated from *Marinobacter* sp.

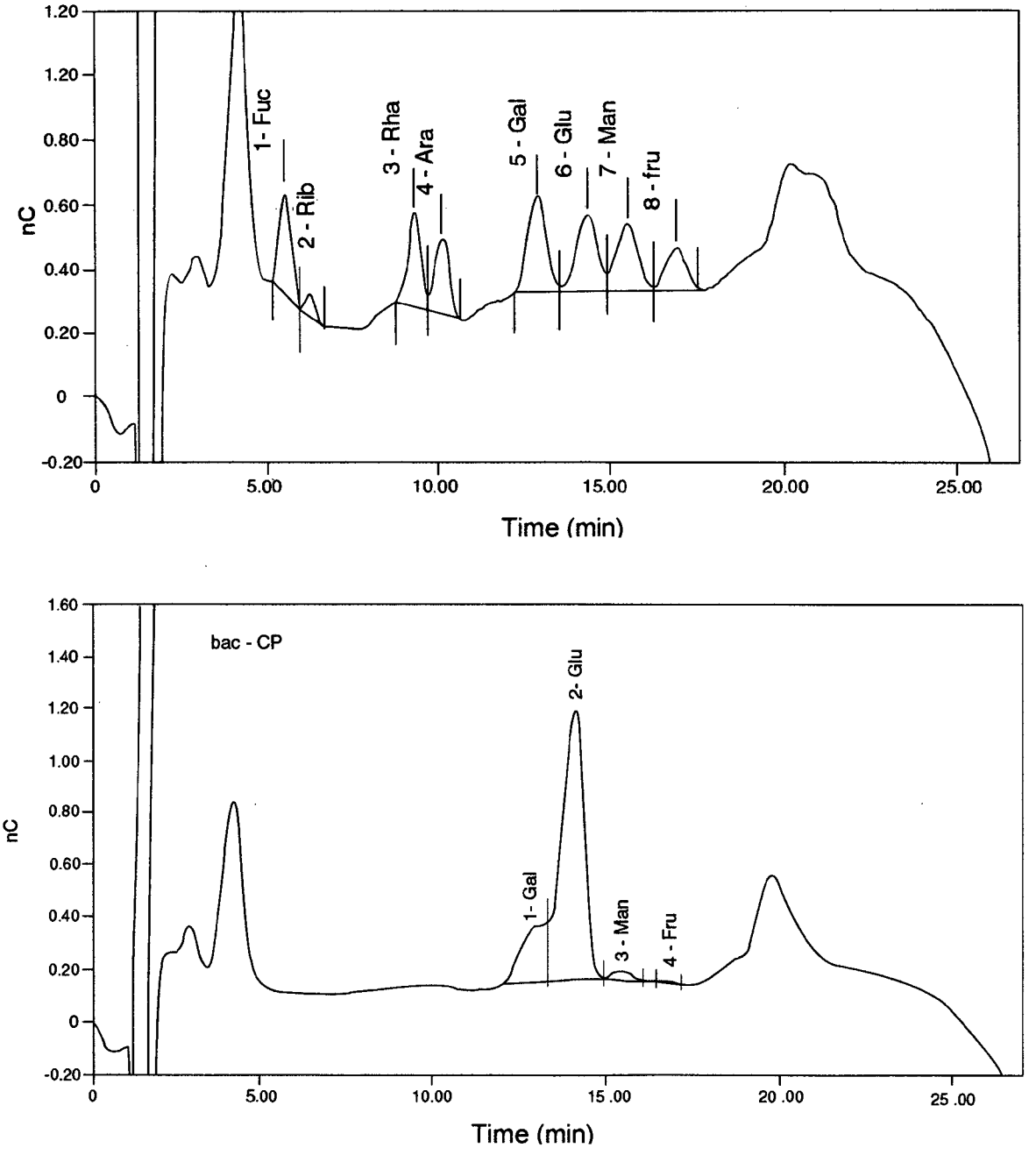

Fig. 3.8 HPLC-PAD chromatographs of a) aldose standard and b) bacterial EPS. A mixture of 8 aldose sugars at 20 nM each was used as a standard for the calibration. The sugars estimated are: I) Fuc- Fucose, ii) Rib- Ribose, iii) Rha- Rhamnose, iv) Ara- Arabinose, v) Gal-Galactose, vi) Glu- Glucose, vii) Man- Mannose/Xylose and viii) Fru- Fructose

Total hydrolysable amino acid (THAA)

The individual amino acid concentration was expressed as mole% and is shown in the **Table 3.6**. Aspartic acid was the major amino acid in the EPS derived from *Marinobacter* and contributed 79.2 mole% to the THAA. This was followed by serine (7.4 mole%) and glutamate (6.9 mole%) (**Fig. 3.9**). Amino acids like glycine/threonine, valine, alanine, phenylalanine and leucine

THAA	Mole %	THAA	Mole%
Aspartate	79.2	β-Alanine	0.07
Glutamate	6.9	Alanine	2.5
Asparagine	0.2	Tyrosine	0.68
Serine	7.4	γ-ABA	0.18
Glutamine	1.6	Methionine	0.02
Histidine	0.5	Valine	1.7
Citrulline	0.2	Phenylalanine	0.68
Glycine/Threonine	2.8	Isoleucine	1.3
Arginine	0.43	Leucine	1.3

Table 3.6: Total hydrolysable amino acid (THAA) composition (mole %) of EPS isolated from *Marinobacter* sp.

contributed around 0.6 to 2.8 mole%. Histidine, citrulline, β-alanine, tyrosine, methionine, isoleucine and γ-ABA were the minor amino acids.

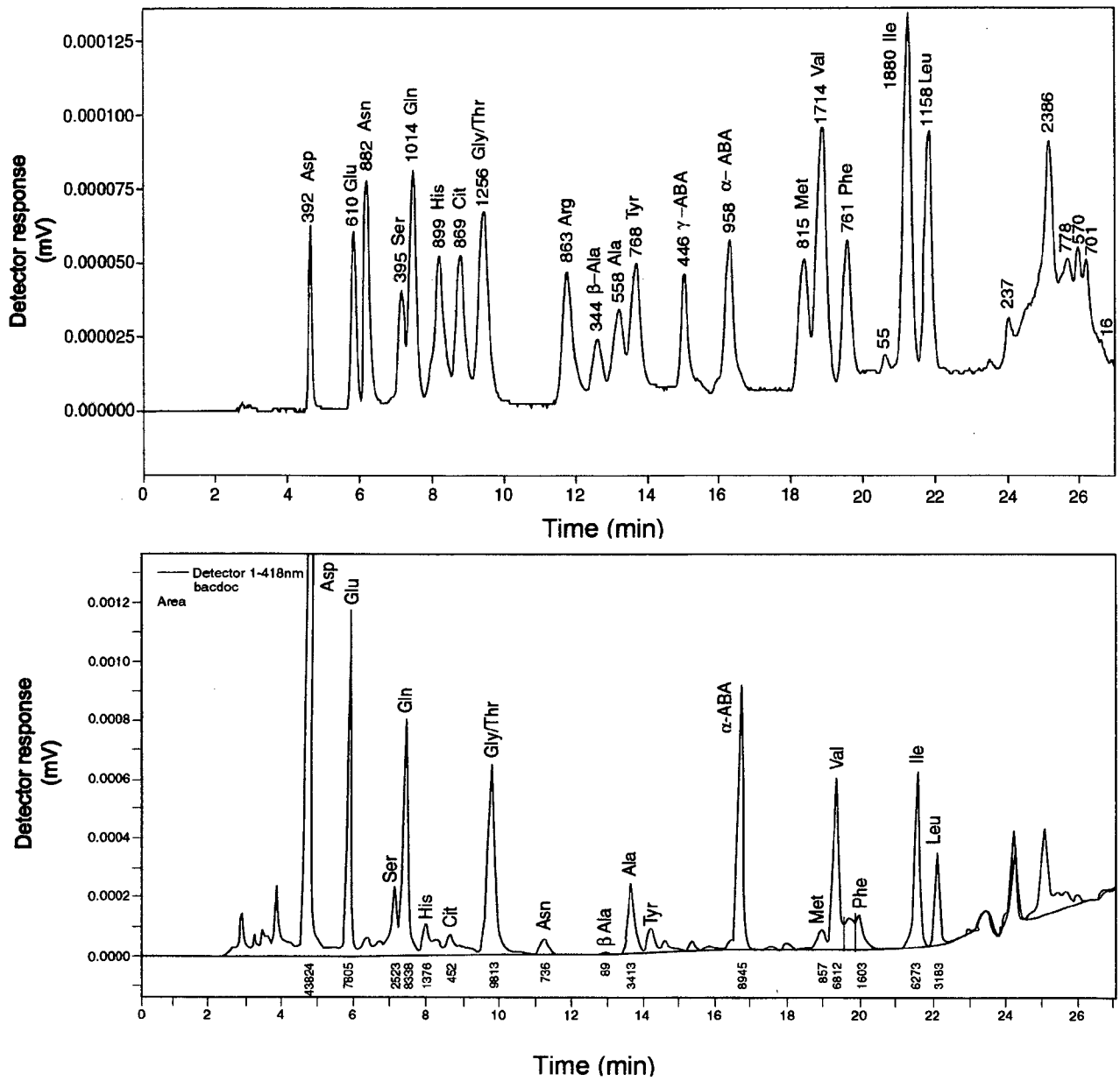

Fig. 3.9 HPLC chromatographs of total hydrolysable amino acids (THAA) in the a) standard and b) bacterial EPS. The standard contained 18 amino acids and α-ABA was used as internal standard. For the list of entire 18 amino acids, please refer to **Table 3.6**.

3.4 Discussion

The bacterial isolate was a gram negative, motile, non-sporulating short rods that grew well in most carbohydrate supplemented medium (**Table 3.2b).** Moreover, the bacterial isolate was facultative aerobe. The characteristics of the bacterial isolate were similar to those of *Marinobacter sp.* belonging to family *Alteromonadaceae* (Gauthier et al 1992, Nguyen et al 1999). Organisms belonging to this genus are gram-negative rods (straight, curved or spiral), non-sporulating having polar flagellum (Balows et al 1992). The members of this genus are strictly aerobes and typically marine in their habitat. *Marinobacter* sp. studied here was isolated from mangrove sediments along the Zuari river, which generally experienced typical marine conditions (higher salinity) during most of the year (Qasim and SenGupta 1977). Like other members of the family *Alteromonadaceae*, *Marinobacter* culture could ferment most sugars without the production of gas but did not grow in presence of carbon source like sorbitol and mannitol. The culture also had the nitrate reducing properties associated with cultures of the family *Alteromonadaceae.* Moreover, the 16s-rRNA sequences showed 99% similarity with that of *Marinobacter sp. Trimyema-2*. Thus both biochemical and molecular methods of identification suggest that the culture was *Marinobacter.* However, unlike other members of the family *Alteromonadaceae* like *Alteromonas, Pseudoalteromonas and Shewanella*, there is no information on the production and characterization of EPS of *Marinobacter sp.*

Various methods including high-speed cold centrifugation (Brown and Lester 1979, Decho 1990), chemical treatments using EDTA (Underwood et al 1995, Bhosle et al 1995), EGTA (Turakhia et al 1983), mild alkali (Bhosle et al 1993, Khandeparker and Bhosle 2001) and sodium chloride (Bhosle et al 1995, Underwood et al 1995) have been reported for extraction of EPS from microbial cultures. Brown and Lester (1979) used only high-speed centrifugation (33000 g) to separate the cells from the EPS. However, it is possible that such high shear may damage the cells and cause contamination of the EPS (Decho 1990). In contrast, treatment of cells with chemicals like EDTA followed by centrifugation has been found to be the most effective extraction method for the isolation of EPS (Decho 1990). We used a combination of both high-speed centrifugation and chemical methods to extract the EPS.

Marinobacter sp. was grown in varying carbon, nitrogen and phosphate concentrations. Microorganisms are known to produce more EPS when grown in carbon enriched medium (high C:N ratios) (Williams and Wimpenny 1977, Sutherland 1983 & 2001, de Souza and Sutherland 1994, Majumdar et al 1999, Nicolaus et al 1999). Limitation of nitrogen is known to trigger increased EPS production by microorganisms (Duguid and Wilkinson 1953, Corpe 1964, Fajon et al 1999). From our data, it was evident that at a constant nitrogen concentration (0.0026% N), the EPS content did not increase much with increasing glucose concentrations. In contrast, bacterial growth increased with glucose concentrations except at 5% glucose concentration (**Fig. 3.1**)

indicating that at higher concentrations, glucose was primarily used to increase the biomass and not for EPS production.

The culture showed a preference for reduced form of nitrogen to support both bacterial growth and maximal EPS production. Moreover, the EPS production was higher when the nitrogen concentrations were low (**Fig. 3.3a & 3.3b**). A similar response in EPS production by *Marinobacter* culture was observed at low phosphate concentrations (**Fig. 3.4**). Increase in EPS production under nutrient limiting growth conditions have been reported in many cultures. For example, optimal EPS production by *Chromobacterium* sp. has been reported when the nitrogen concentrations in the medium were low (Duguid and Wilkinson 1964). Similarly, increase in EPS yield has been reported for *Pseudomonas* PB1 under minimal nitrogen and phosphate concentrations (Williams and Wimpenny 1977). Although not much is known about the mechanism regulating the production of EPS in nutrient depleted conditions, a shift in the biosynthetic pathways might explain the observed changes.

In contrast to the limitation of nitrogen and phosphates, limitations of other essential elements like magnesium has relatively more influence on the composition of the EPS than its production (Davidson 1978). For example, presence of Mg^{2+} causes gelling of bacterial polymer (Smith et al 1961). Similarly, magnesium ions can help in stabilizing the polymer structure (Characklis and Cooksey 1983). In the present study, EPS increased marginally with the increase in magnesium concentrations suggesting that

increase in magnesium concentrations did not improve the production of the polymer.

The production of the EPS during the growth of *Marinobacter* had certain distinct features. For example, maximum EPS was produced in the late exponential growth phase of the culture. Moreover EPS production increased rapidly within the first 20 h of culture growth and increased gradually up to 100 h. The EPS production was maximal as the culture approached stationary phase. Bacteria produce EPS during their log-phase depending upon the growth conditions and age of the culture (Decho 1990). *Marinobacter* sp. in the present study appears to produce capsular form of EPS. The decline in EPS concentrations at the end of the incubation was however not explainable by cell density variations (which did not vary much). It is possible that EPS production was less in the stationary phase of the bacterial growth, there by reducing the EPS concentrations in the medium.

EPS produced during different phases of growth have specific properties and functions. For example, capsular form of EPS is generally produced during log phase that forms a tight envelope around the cell, promoting cell attachment to substratum (Carlsson 1967, Costerton 1984). Capsules may also provide better protection against predation and help bacteria survive in low pH (Decho and Lopez 1993). Bacterial capsules also act as a good metal adsorbent (Brown and Lester 1982, Schlekat et al 1998) and may act as a buffer against metal toxicity. Thus the production of EPS in the exponential phase has

ecological significance for the organism and may play an important role during the growth of *Marinobacter* sp.

The functions and the properties of the EPS vary due to differences in their chemistry. The bacterial EPS had lesser carbohydrate (TCHO) content compared to proteins (**Table 3.3**). The high protein content is also reflected in the relatively low C:N ratios of the polymer. The TCHO contribution to the total EPS was lower than those reported by Rougauex et al (1996) (42%), Bejar et al (1998), Guezennec et al (1998) (57%) and Majumdar et al (1999) (51%). However, a similar low TCHO content has been reported for the EPS isolated from marine *Pseudomonas* sp. (11.5 to 13.5 μg mg^{-1} EPS) (Beech et al. 1999). The protein content of *Marinobacter* EPS was 22.5% by weight, which was similar to the EPS produced by *Vibrio* sp. (Majumdar et al 1999). Proteins in the microbial EPS play an important role in stabilizing the tertiary polymer structure and to some extent help in adhesion of cells to surfaces (Decho 1990, Hoagland 1993, Wingender et al 1998). It is possible that the proteins present in the bacterial EPS might be important for the stability of the EPS.

Carboxylated sugars like uronic acid formed important fraction of bacterial EPS. The uronic acid content of *Marinobacter* EPS was 17.5% of the polymer by weight (**Table 3.3**). This was relatively greater than EPS of fouling bacteria like *Halomonas* strains (0.6-11.1%, Bejar et al 1998) and *Vibrio* (14.1%) (Majumdar et al 1999) but lower than that of Rougauex et al (1996) (38%). The FTIR-spectrum of the bacterial EPS also showed definite peaks for carboxylates at 1029.9 and 1107.1 cm^{-1} (**Fig. 3.7**). The presence of such

functional groups gives a general idea about the nature of the EPS. For example, uronic acid imparts an overall negative charge and reduces the pH of the polymer (Corpe 1970). Similarly, uronic acid may also affect the solubility of the polymer and may help in metal-complexation (Kaplan et al 1988). The presence of relatively higher uronic acid content in the EPS indicates that the bacterial EPS may be useful in binding of metals and therefore might find industrial application.

On the other hand, the non-sugar component like sulphates was relatively low in the EPS produced by *Marinobacter* (2.7%; **Table 3.3**). This value was less than that of EPS isolated from *Alteromonas infernus* (8.8%; Guezennec et al 1998) and *Alteromonas* EPS (5%; Rougauex et al 1996). The presence of sulphates was also observed in the FTIR-spectrum of the bacterial EPS (**Fig. 3.7**). Sulphate moieties provide flexibility to EPS, impart gel-like consistency, prevent desiccation and help to stabilize the polymer (Hoagland et al 1993). It is possible that sulphates present in bacterial EPS might be useful in flocculation and aggregation of cells. Such aggregation might help the bacterium survive nutrient stress (Hoagland et al 1993).

The bacterial exopolymer was a heteropolysaccharide as observed from the sugar (aldoses) composition. Glucose was the most dominant aldose in the EPS followed by galactose (**Table 3.4**). The glucose contribution in the *Marinobacter* EPS was similar to that of *Vibrio* (75%, Majumdar et al 1999) but was greater than those reported for *Alteromonas macleodii* (11.2 to 14.8%, Rougeaux et al 1996) and *Alteromonas infernus* (1 to 16% glucose,

Guezennec et al 1998). The absence of deoxy sugars in the *Marinobacter* EPS was similar to those observed in the EPS isolated from *Alteromonas infernus* (Guezennec et al 1998), a cyanobacterium (*Cyanothece sp.*, Philippis et al 1998) and *Vibrio* sp. (Majumdar et al 1999). *Marinobacter* EPS being enriched in glucose might serve as a carbon source for heterotrophs in the marine environment.

The relatively higher concentrations of proteins in bacterial EPS was also reflected in their amino acid concentrations. Interestingly, aspartic acid was the major amino acid observed in the EPS forming 79.2% of total amino acids. Other major amino acids like serine, glutamate, glycine/threonine, alanine, leucine and valine formed 6.1% to 24.7% of the THAA concentrations (**Table 3.5**). The high aspartate concentration in the EPS was intriguing and could not be explained. It is possible that the amino acids like aspartic acid might belong to high molecular weight proteins involved in the structural organization of the EPS.

3.5 Conclusions

In conclusion, EPS production by *Marinobacter* sp. was influenced by physiological factors and medium composition. Bacterial polymer was nitrogen enriched as observed from the low C:N ratios, which is reflected in its higher protein content. The bacterial EPS was high molecular weight heteropolysaccharide with glucose as the principal component of monosaccharides. *Marinobacter* sp. produced sulphated EPS having a fair

amount of uronic acid. Such compounds in the EPS might assist in cell attachment, motility and provide protection against metal toxicity and desiccation.

CHAPTER 4

Role of microbial EPS in macroaggregate production

Chapter 4

4.1 Introduction

Macroaggregates are particles that may range from 500 µm to more than 10 cms in length (**Table 4.1**, Simon et al 2002) and occur in varying forms such as amorphous aggregates, stringers, flocs, mats etc (Herndl and Peduzzi 1988, Stachowitsch et al 1990). Macroaggregates form an important constituent of both suspended and sedimenting particles in aquatic environments (Silver and Alldredge 1981, Alldredge and Gotschalk 1989, Eisma et al 1983, Reibesell 1991). Unlike solid particles, macroaggregates are porous, hydrated, and fractal in nature (Logan and Wilkinson 1990, Alldredge and Jackson 1995, Chen and Eisma 1995, Jackson et al 1997). The

Type of organic matter	Size range
Macroaggregates (marine snow)	0.5 mm and above
Microaggregates	1 to 500 µm
Algae/cyanobacteria	1 to 100 µm
Bacteria	0.1 to 5 µm
Submicron particles	0.05 to 1 µm
Colloids	0.004 to 1 µm
Proteins/Carbohydrates	$< 10^4$ to 10^6 daltons

Table 4.1: Size spectra of the major organic matter constituents in marine ecosystems.

macroaggregates may contain live cells of phytoplankton, bacteria and protists, cell debris, abandoned larvacean houses and pteropod nets, fecal matter etc entrapped in a matrix of exopolysaccharides (EPS) (Alldredge and Silver 1988, Alldredge and Gotschalk 1989, Herndl 1993, Kiørboe 2001, Simon et al 2002).

The macroaggregates play an important role in biogeochemical cycling of elements, life-cycle of micro-organisms and aquatic trophic-web. For example, phytoplankton cells form macroaggregates under nutrient depleted conditions and sink into deeper waters to escape growth-limiting conditions. These aggregates may act as seed-banks for future blooms and hence plays an important role in the life-cycle of phytoplankton like diatoms (Smetacek 1985). While sedimenting as marine/lake snow, these aggregates act as a vehicle for the vertical transport of organic matter to the benthos (Honjo 1982, Alldredge and Gotschalk 1989, Herndl 1993, Passow et al 2001). Aggregates may also serve as food for grazers like zooplankton (copepods, decapods), krills etc. (Alldredge and Silver 1988, Biddanda and Pomeroy 1988, Herndl 1993, Kiørboe 2001). On the other hand, these particles are also colonized by bacteria and may serve as sites for bacterial mediated mineralisation of organic matter and nutrient regeneration (Azam and Cho 1987, Kirchman and Ducklow 1987, Biddanda 1988, Cho and Azam 1988).

The production of macroaggregates in the natural environments involves collision and sticking together of smaller particles following biotic and abiotic processes. In a biotic process, microorganisms (few microns in length) may act as fine suspended particles that might stick to each other directly i.e. cell-

cell (Engel and Schartau 1999) or aggregate with the help of exopolysaccharides (EPS) produced during its growth to form larger particles (Paerl 1974, Biddanda 1985, Herndl 1993, Decho and Herndl 1995). In contrast to biologically mediated processes, particles might also be produced directly from high molecular weight dissolved organic matter (DOM) by purely abiotic processes including 1) self-coagulation (Kepkay 1994, Mari and Burd 1998, Chin et al 1998, Passow 2000) and 2) bubble adsorption (Mopper et al 1995, Zhou et al 1998, Mari 1999). Similarly, the adsorption of the DOM by charged non-living particles may also lead to the production of aggregates (Gerritsen and Bradley 1987, Eisma 1993).

Microorganisms like bacteria and phytoplankton produce sticky, high molecular weight, surface-reactive EPS that form an important constituent of the DOM in the aquatic environments (Decho 1990). Both field and laboratory observations have shown that EPS released by microorganisms are involved in both biotic and abiotic production of macroaggregates (Kiørboe and Hansen 1993, Passow et al 1994, Schuster and Herndl 1995, Alldredge et al 1995 & 1998, Mopper et al 1995, Mari and Burd 1998, Engel 2000, Passow 2000 & 2002). The presence of microbial EPS influences the aggregation process by altering the surface characteristics and the sticking coefficient of the colliding particles (Kiørboe and Hansen 1993, Engel 2000). Microbial EPS also act as precursors for the production of a class of transparent exopolymer particles or TEP, which is considered important for aggregation (Mopper et al 1995, Stoderegger and Herndl 1998, Passow 2002). Similarly, Coomassie blue stained particles (CSP) are another class of organic particles that are

predominantly rich in proteins and as abundant as TEP in the marine environments (Azam and Long 1996). However unlike TEP, nothing is known about the role of CSP in particle aggregation.

Field and laboratory experiments have reported on the role of EPS in aggregation (Kiørboe and Hansen 1993, Passow et al 1994, Passow and Alldredge 1995, Schuster and Herndl 1996, Passow et al 2001, Prieto et al 2001) and the production of transparent exopolymeric particles (TEP) (Mari and Burd 1998, Stoderegger and Herndl 1998, Passow 2002). Most of the studies on aggregation has been conducted during the growth of micro-organisms (Alldredge and Gotschalk 1989, Passow and Alldredge 1995) and there is little information on the production of macroaggregates (visible particles) from dissolved EPS in the absence of growing cells (Stoderegger and Herndl 1998). On the other hand, production of EPS does not necessarily warrant the production of aggregates (Kiørboe and Hansen 1993), suggesting that changes in EPS chemistry can influence the production of macroaggregates in the natural environments. However, there is little information on the influence of the chemical composition of the EPS on the production of macroaggregates.

In this chapter, an attempt is made to study the production of macroaggregates from EPS isolated from two different sources. The study was carried out primarily to assess the variations in macroaggregate size and abundance over time. Apart from abundance of macroaggregates and its size variation, the changes in the concentration of organic particles like TEP and

Coomassie blue stained particles (CSP) and their role in regulating the aggregate abundance and size are also addressed.

4.2 Material and Methods

4.2.1 Production and isolation of Marinobacter EPS

Pure culture of *Marinobacter* was grown in 2 l flasks containing 1 l of BSS medium (pH 7.6) (**Appendix Ib**) over 14 days at 30 ± 2°C. The medium was supplemented with 3% glucose, low molecular weight fraction of yeast extract (0.05% final concentration) and peptone (0.1% final concentration). The details of isolation of EPS from the culture are given in Chapter 3.

4.2.2 Production and isolation of EPS from Skeletonema costatum

Axenic culture of *Skeletonema costatum* Greve was grown in 6.5 l of Guillard's f/2 medium prepared in aged 0.22 µm filtered seawater (Appendix II). The medium was distributed in 2 l capacity Schott Duran screw capped bottles, autoclaved and inoculated with axenic cells of *Skeletonema costatum* (6.3×10^5 ml^{-1}). The axenic cultures were grown under 12 h light: 12 h dark conditions at 15 ± 0.5°C for 27 days. At the end of the incubation period, the culture was centrifuged at 3000 rpm (7800 g) at 4°C to separate the EPS from the cells. The supernatant was filtered through pre-ashed GF/F filters and then concentrated by passing through a 3 kDa MW spiral membrane filter attached to a tangential flow filtration unit. The low molecular weight fraction of the medium was discarded and the higher molecular weight fraction

concentrate (retentate) was reduced in volume to < 50 ml at 35°C using a rotary evaporator. The diatom EPS was then filtered through 0.22 µm filters and precipitated overnight in absolute ethanol at 4°C. The precipitate was then redissolved in small quantities of deionised water and dialysed against deionised water through 8kDa MW dialysis bags. Once dialyzed, the EPS was reduced in volume, lyophilized and stored at –20°C.

4.2.3 Chemical characterization

The details of the various chemical analyses carried out on *Marinobacter* EPS are given in Chapter 3. EPS isolated from *S. costatum* was also analysed for its bulk chemistry including total carbohydrates, proteins, uronic acid, sulphates and monosaccharide composition following the methods discussed in Chapter 3.

4.2.4 Production of macroaggregates from EPS

In order to produce macroaggregates, four sterile rolling tanks (1.5 l capacity) were filled completely with sterile f/2 medium (Appendix-II). To one tank, nothing was added and was treated as negative control (*NC*) tank. To the second tank, killed *Marinobacter* cells were added and was treated as killed-bacterial control (*KB-C)* tank. To the third tank *(Eps)*, *Marinobacter* EPS (300 µM-C final concentration) was added to the medium while to the fourth tank (*Eps + KB*) killed *Marinobacter* cells were added along with 300 µM-C (final concentration) of *Marinobacter* EPS. The third and fourth tanks were used as experimental tanks. In another experiment, all the experimental conditions

were kept same except that EPS derived from *S. costatum* was used as DOM in place of *Marinobacter* EPS. All rolling tanks were incubated in the dark and rolled at a speed of 2 revolutions per minute on a rolling table (Grossart et al 1998).

Image analyses

Prior to every sampling, images of the samples were captured for 30 s using a Sony HAD CCD handy cam (680K gross pixels) while illuminating the tanks with side-arm focus lamps. In order to analyze the images, a 250x250 pixel window (1 pixel= 60 µm) was chosen and the images were gray-scale transformed to highlight the aggregates within each window (**Plates I & II**). The transformed images were then used to analyze the particle abundance and size using SISview image analyzing software. In each image, aggregates that were clearly focused were selected for abundance and size distribution analyses. The equivalent spherical diameter (ESD) was first calculated using the image analyzing software and used to estimate the size of the aggregates expressed as equivalent spherical area (mm^2). The aggregates were then grouped into different size classes ranging from 0.2 mm^2 to 100 mm^2. The particle abundance was estimated by summing up the particle numbers in each category to give total abundance. The particle size was the mean size of all the aggregates.

4.2.5 Macroaggregate sampling

A known volume (8 ml) of the sample was removed at regular intervals. Equal volume of sterile medium was added to the tanks so as to maintain the volume of medium in the rolling tanks. Duplicate samples (2 ml each) of macroaggregates were filtered on to polycarbonate membrane filters (5 µm pore size) (Grossart et al 1998) under vacuum (< 10 mbar pressure) to enumerate TEP and CSP. The TEP samples were stained with 0.22 µm prefiltered 0.02% Alcian blue prepared in 0.06% glacial acetic acid (pH 2.5; Alldredge et al 1993). Similarly, CSP samples were stained with 1 ml 0.04% Coomassie brilliant blue G-250 stain (Long and Azam, 1996). The samples were then filtered dry, placed over a drop of oil on a glass slide and enumerated under 100x and 200x total magnification.

4.2.6 Statistical analyses

A simple regression analysis was carried out to assess the relationships between various parameters. One-way ANOVA for unequal data sets was used to assess the differences in abundance of aggregates, TEP, CSP and size of aggregates produced in all the experiments. The statistical analyses were carried out using Statistica 5.0 software installed in the personal computer.

4.3 Results

4.3.1 Chemical composition of EPS derived from Marinobacter sp. and S. costatum

The bulk chemical and molecular composition of *Marinobacter* EPS have been discussed in Chapter 3 (**Table 3.4 and 3.5**). The bulk chemical composition of the EPS derived from *Skeletonema costatum* is shown in **Table 4.2a**. The diatom EPS contained relatively higher concentrations of sulphates and lesser amounts of protein and uronic acids compared to *Marinobacter* EPS. The monosaccharide composition showed the heterogeneity of the diatom EPS (**Table 4.2b**). Glucose was the predominant monosaccharides in the EPS (53.3 mol%) followed by mannose + xylose (16.8 mol%) and galactose (16.5%) (**Table 4.2b**).

Parameters	Concentration (µg/mg)
TCHO	28.3
Proteins	35.8
Uronic acid	46.2
Sulfates	215.4
C:N (w:w)	8.1

Table 4.2a: Bulk chemical composition of EPS isolated from *S. costatum*.

Monosaccharide composition	Concentration (mole %)
Fuc	2.7
Rib	--
Rha	6.2
Ara	--
Gal	16.5
Glu	53.3
Man+Xyl	16.8
Fru	4.6

Table 4.2b: Monosaccharide (Aldose) composition (mole%) of EPS isolated from *S. costatum.*

4.3.2 Macroaggregate abundance (numbers cm^{-2})

In experiment-I wherein *Marinobacter* EPS was used as dissolved organic carbon, the particle abundance in tank containing only EPS (*Eps*) decreased from 53 cm^{-2} to 25 cm^{-2} in the first 20 h followed by an increase to 61 aggregates cm^{-2} after 70 h (**Fig. 4.1a**). The particle abundance declined thereafter until the end of the experiment. On the other hand, no large-scale temporal variations could be observed in the abundance of particles in tank having both *Marinobacter* EPS and killed *Marinobacter* cells (*Eps + KB*) (**Fig. 4.1a**). The abundance of particles varied from 1.8 to 8.9 aggregates cm^{-2} (**Table 4.3**). In contrast to the experimental tanks, the particle counts in tank having only medium (*NC*) did not vary much up to 99h and declined thereafter

(**Fig. 4.1a**). Similarly, particle abundance in tank containing only killed bacterial cells (KB-C) also showed small-scale variation (**Fig. 4.1a**) and ranged from 11 to 27.1 aggregates cm^{-2} (**Table 4.3**).

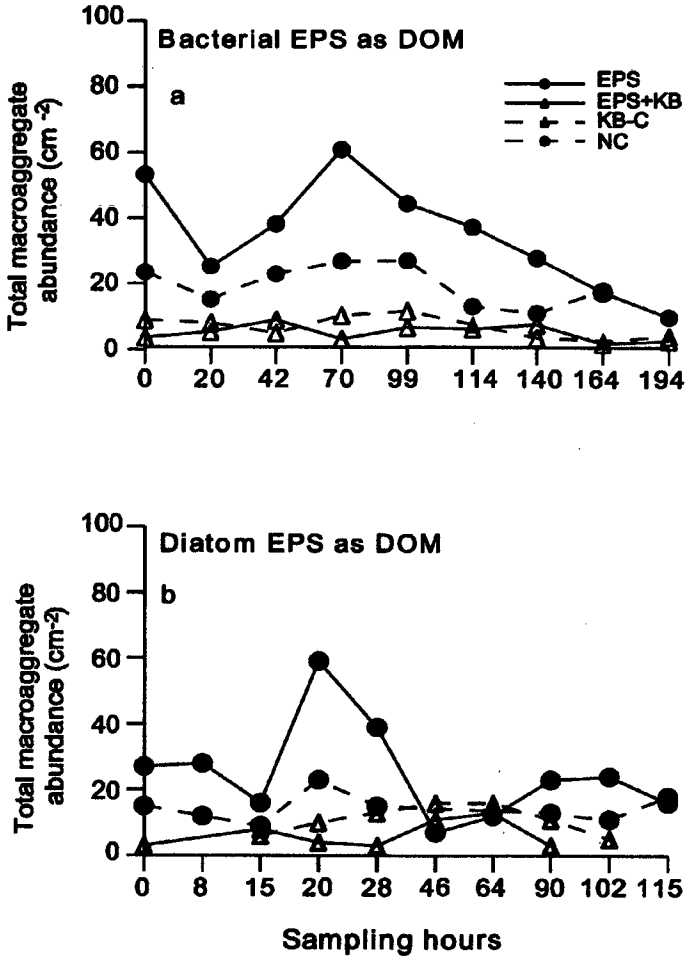

Fig. 4.1 Changes in the abundance of macroaggregates produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM .

In experiment-II where *S. costatum* EPS was used as DOM, the abundance of particles in the tanks having only diatom EPS increased from 28 aggregates cm^{-2}, reached a high (59 aggregates cm^{-2}) after 20 h and declined thereafter (**Fig. 4.1b**). Similarly, the particle abundance in the tank containing both diatom EPS and killed *Marinobacter* cells varied from 3 to 13 aggregates cm^{-2}

(**Table 4.3**) and did not show any specific trend over the period of incubation (**Fig. 4.1b**). On the other hand, the total abundance of aggregates in the tank containing only medium ranged from 9 to 23 aggregates cm^{-2} (**Fig. 4.1b**) and increased till 64 h followed by a decline.

4.3.3 Macroaggregate size (mm^2)

In experiment I, the aggregate size in the tank having *Marinobacter* EPS increased from 1.2 mm^2 in the beginning to 16 mm^2 within 20h of incubation, declined to 7 mm^2 and reached the maximal (22.7 mm^2) by the end of the experiment (**Fig. 4.2a) (Plate I)**. On the whole, the aggregate size ranged from 1.1 to 22.7 mm^2 (**Table 4.3**). In contrast, the aggregate size in the tank containing both *Marinobacter* EPS and killed bacterial cells, the aggregate size increased only after 99 h of incubation (**Fig. 4.2a**). The aggregate size in this tank ranged from 0.66 mm^2 to 13.2 mm^2 during the course of the experiment (**Table 4.3**). In contrast, the control tanks (both *NC* and *KB-C*) did not show the formation of macroaggregates except near the end of the experiment (**Fig. 4.2a**). The aggregate size ranged from 0.5 to 9.5 mm^2 and 0.6 to 2.9 mm^2 in the tank having only medium and killed bacterial cells, respectively **(Table 4.3)**.

In the second experiment, the macroaggregate size in the experimental tank containing only diatom EPS increased from 1.6 mm^2 to 31.6 mm^2 only after 28 h, increased marginally till 102 h (34.5 mm^2) and then decreased to 19.4 mm^2 (**Fig. 4.2b**). On the other hand, no such increase in the macroaggregate size

was observed in the tank with EPS and killed bacterial cells (**Fig. 4.2b**). Similarly, the particle size in the control tanks having only medium and killed bacterial cells varied from 0.15 mm^2 to 1.3 mm^2 and 0.07 mm^2 to 1.03 mm^2, respectively and did not show any large-scale variations (**Table 4.3**).

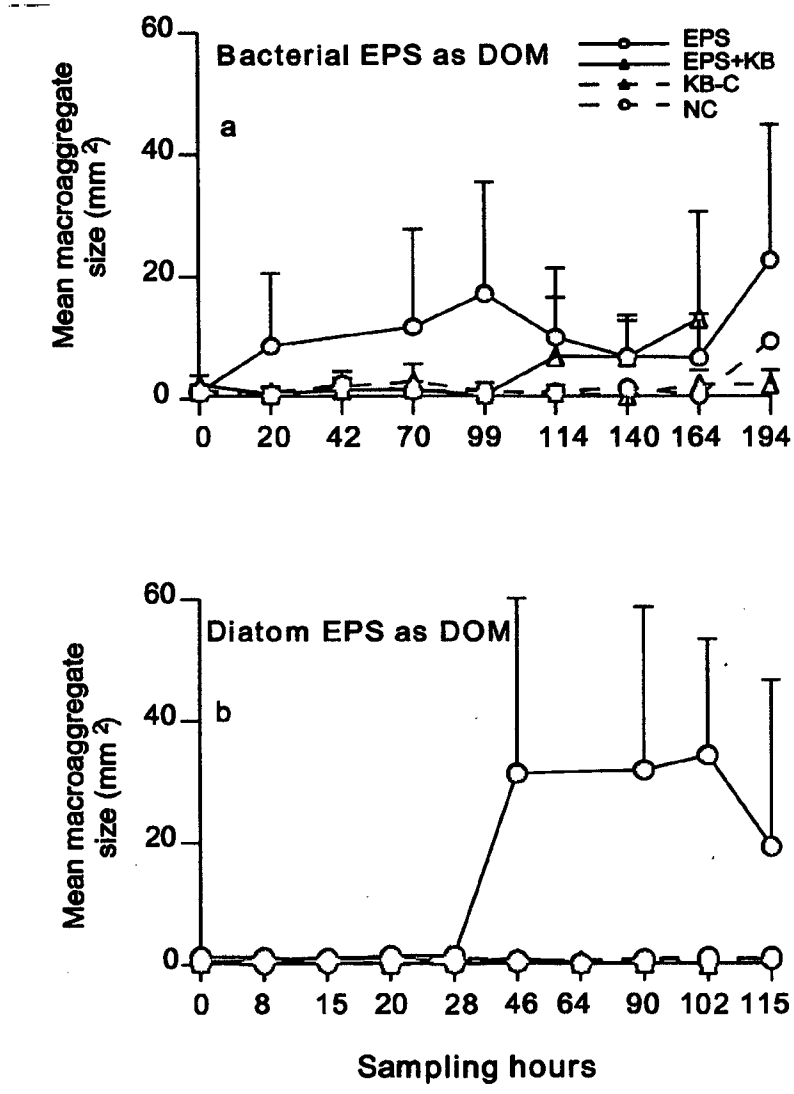

Fig. 4.2 Changes in the mean size of macroaggregates produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM. The error bars indicate the standard deviation.

4.3.4 TEP and CSP abundance (ml^{-1})

In experiment-I, the abundance of TEP and CSP declined with time in both experimental and control tanks. The abundance of TEP in the tank containing only bacterial EPS increased from 0.6 x 10^3 ml^{-1} to a high of 5.7 x 10^3 ml^{-1} within 20 h followed by a decrease thereafter till the end of the experiment (**Fig. 4.3a**). A similar variation was observed in the abundance of CSP produced in this tank (**Fig. 4.4a**) that varied from 0.6 to 3.7 x 10^3 ml^{-1} (**Table 4.3**). The abundance of TEP in tank containing bacterial EPS and killed

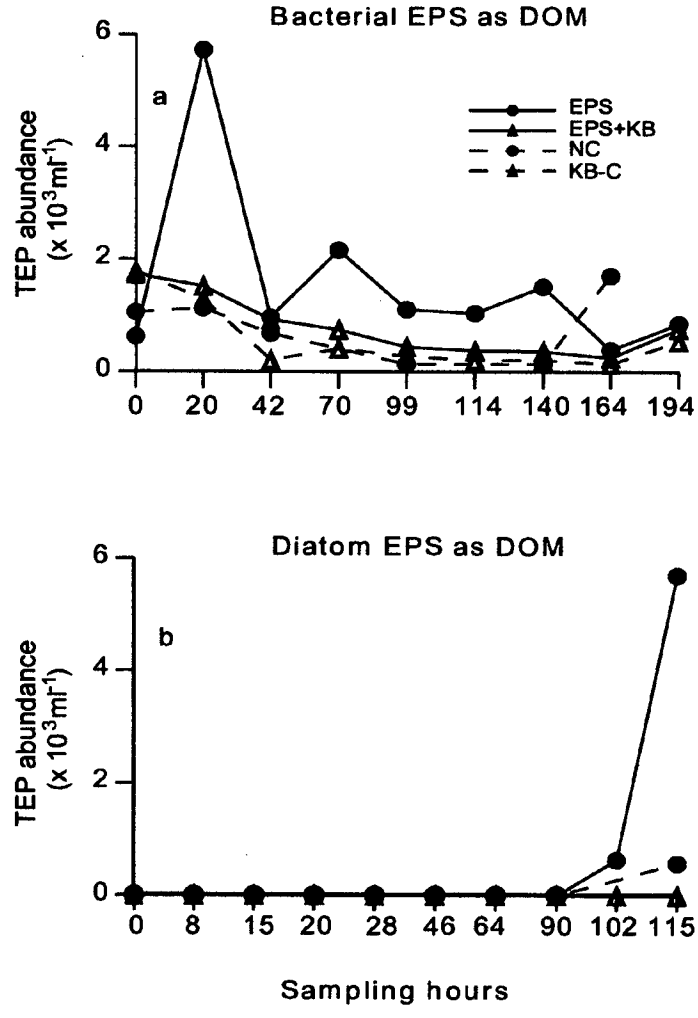

Fig. 4.3 Changes in the abundance of TEP produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM.

Table 4.3: Range of the particle size, abundance of particles, TEP, and CSP produced during the aggregation experiment using two sources of EPS.

Experiments	DOM source	Tanks	Treatments	Particle abundance	Particle size	TEP abundance	CSP abundance
				Range (cm^{-2})	Range (mm^2)	Range (x 10^3 ml^{-1})	Range (x 10^3 ml^{-1})
I	Bacterial EPS	Control	Only medium (NC)	11-27.1	0.5-9.5	0.13-4.6	0.13-2.4
			Killed bacteria (KB-C)	2.7-11.6	0.6-2.9	0.2-1.7	0.13-1
		Experimental	EPS only (Eps)	10-60.9	1.2-22.7	0.4-5.7	0.6-3.7
			With killed bacteria (Eps + KB)	1.8-8.9	0.7-13.2	0.24-1.7	0.3-2.5
II	Diatom -EPS	Control	Only medium (NC)	11-23	0.14-1.3	0-1.77	0-3.7
			Killed bacteria (KB-C)	5-16	0.08-1.3	<0.05	<0.05
		Experimental	EPS only (Eps)	7-59.7	0.6-34.5	0-5.7	0-5.3
			With killed bacteria (Eps + KB)	3-13.0	0.3-1.2	< 0.05	<0.05

bacterial cells declined from 1.7 x 10^3 ml^{-1} at 0 h to 0.24 x 10^3 ml^{-1} after 164 h of incubation (**Table 4.3**). On the other hand, the CSP abundance in the same tank also declined with time (**Fig. 4.4a**) from 2.9 x 10^3 ml^{-1} to 0.3 x 10^3 ml^{-1} (**Table 4.3**). In contrast, abundance of TEP and CSP in the tank containing only medium decreased from 1.1 x 10^3 ml^{-1} on day 0 to almost 0.3 x 10^3 ml^{-1} (**Fig. 4.3a**) and 1.1 x 10^3 ml^{-1} to 0.1 x 10^3 ml^{-1} (**Fig. 4.4a**) by the end of the experiment. On the other hand, TEP counts in tank having killed bacterial cells also decreased from 1.8 x 10^3 ml^{-1} on day 0 to 0.1 x 10^3 ml^{-1} at the end of the experiment (**Fig. 4.3a**). Moreover, the CSP counts also declined from 2.9 to 0.1 x 10^3 ml^{-1} with time (**Fig. 4.4a**).

In experiment-II, the abundance of TEP in tank having only diatom EPS was noticeable only at the end of the experiment (**Fig. 4.3b**) and varied from 0 to 5.7 x 10^3 ml^{-1} (**Table 4.3**). In contrast, the abundance of CSP in the tank increased after 8 h of incubation and reached a high of 4.7 x 10^3 ml^{-1} after 20 h, declined to 2.6 x 10^3 ml^{-1} after 64 h and was maximal at the end of incubation period (**Fig. 4.4b**). The abundance of TEP and CSP in tank having both killed bacterial cells and diatom EPS was negligible (< 0.05 x 10^3 ml^{-1}) (**Fig. 4.4b**) with very little variations over time. In contrast, the TEP counts in the tank having only medium did not vary much and remained <0.05 x 10^3 ml^{-1} except for a small increase at the end of the experiment. However, the CSP counts in the same tank showed a slight increase with time and ranged from 0 to 3 x 10^3 ml^{-1} during the course of the experiment. The abundance of TEP and CSP in tank containing killed bacteria was negligible (**Fig. 4.3b and 4.4b**).

4.3.5 Statistical analyses

In order to assess the relationship between various parameters, Pearson's correlation coefficient was carried out. There was no correlation of macroaggregate size and abundance with TEP and CSP. Macroaggregate size had no specific trend with particle abundance (**Table 4.4**). The

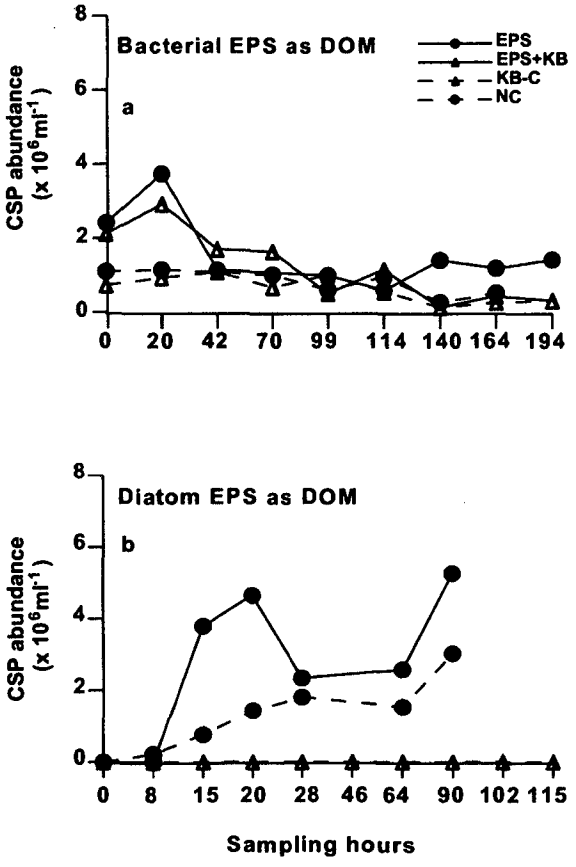

Fig. 4.4 Changes in the abundance of CSP produced in experimental and control tanks having bacterial EPS (a) and EPS of diatom (b) as sources of DOM.

abundance of TEP had no relation with macroaggregate size except for positive trend in *KB + Eps* tank of experiment-I. On the other hand, TEP abundance had a linear trend with particle abundance. The abundance of

Table 4.5: One-way ANOVA for unequal size class carried out on the data to explain the differences in the abundance of macroaggregates, TEP, CSP and the size of the macroaggregates produced during the aggregation experiment.

Treatments		Only EPS			EPS + killed bacterial cells			Only medium			Medium+ killed bacterial cells		
Parameters		SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Aggregate Abundance	Between expts.	1.62	1.62	28.1***	0.08	0.08	2.80	4245.8	4245.8	76.0***	1256.3	1256.3	96.2***
	Within expts.	1.04	0.04		0.41	0.03		947.1	55.7		195.8	13.05	
	Total	2.66			0.49			5193.9			1452.1		
Aggregate size	Between expts.	2105.8	2105.8	9.15**	88.9	88.9	6.76*	43.3	43.3	12.1*	26.4	26.4	73***
	Within expts.	3684.14	230.26		223.5	13.15		64.5	3.6		5.8	0.36	
	Total	4079.29			312.5			107.8			32.2		
TEP counts	Between expts.	96.33	96.33	615.6***	43.16	43.16	29.52***	3.60	3.60	22.9**	2.54	2.54	16.99*
	Within expts.	2.82	0.31		26.32	1.46		2.20	0.15		2.69	0.15	
	Total	99.15			69.48			5.80			5.23		
CSP counts	Between expts.	89.97	89.97	22.23***	48.44	48.44	39.42***	14.5	14.5	26.5**	3.31	3.31	63.8***
	Within expts.	60.70	4.05		22.12	1.23		7.1	0.55		0.93	0.05	
	Total	150.67			70.56			21.6			4.24		

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

CSP did not appear to have any correlation with macroaggregate size and abundance.

In order to assess the effect of EPS derived from different source on the production of macroaggregates, TEP and CSP, a One-way ANOVA for unequal data set was carried out (**Table 4.5**). Significant differences in the variance of particle abundance and size were observed when aggregates were produced using EPS derived from two different sources. For example, the amount and size of macroaggregates produced in tank having bacterial EPS (experiment I) was significantly different ($p < 0.001$) from that of tank containing diatom EPS (experiment II). However, significant differences were observed only in the abundance of TEP and not in the abundance of CSP. Similarly, aggregates produced in *Eps* + *KB* tanks in the two experiments were significantly different in the abundance of macroaggregates, TEP and CSP and the macroaggregate size ($p < 0.001$ and $p < 0.005$). The controls (both *NC* and *KB*) showed significant differences ($p<0.01$ and $p < 0.001$) in their abundance of macroaggregates, TEP and CSP. However, only moderate differences ($p<0.05$) were observed in the size of the macroaggregates between the *NC*-tanks.

4.4 Discussion

Various methods including rolling tanks, bubbling and couvette flocculators have been used to study the production of TEP and aggregates. Shanks and Edmondson (1989) were the first to use the rolling tanks to generate marine-

Table 4.4: Correlation matrix of particle abundance, size and TEP abundance produced using bacterial and diatom EPS in the experimental and control tanks.

Parameters		Experiment I			Experiment II		
Tanks		Size	TEP	CSP	Size	TEP	CSP
Eps	Abundance	-0.08	**0.81****	-0.36	-0.32	0.31	0.17
	Size		-0.04	0.26		0.085	0.16
KB+Eps	Abundance	-0.34	-0.06	0.45	0.42	--	--
	Size		0.47	-0.20		--	--
NC	Abundance	0.05	**0.09**	0.28	0.11	0.29	0.14
	Size		-0.60	-0.51		0.09	0.39
KB-C	Abundance	0.13	**0.34**	0.65	0.46	--	--
	Size		-0.19	0.04		--	--

*: $p<0.05$; **: $p< 0.01$; ***: $p< 0.001$
--: Since the TEP and CSP counts were negligible, correlations were not carried out in this experiment.

snow like aggregates from unfiltered seawater. Since then, rolling tanks have been widely used to produce aggregates from laboratory cultures (Reibesell 1991) and natural limnic and marine waters (Artolozaga et al 1997, Grossart et al 1998, Unanue et al 1998). In this method, shear is generated in the liquid thereby causing collisions of aggregates. Upon collision, small aggregates are formed first that produce larger aggregates by differential settling. Although

this technique does not completely mimic natural conditions, the method is preferred largely for the convenience of sampling and image analyses (Unanue et al 1998, Simon et al 2002). Moreover, the characteristics of aggregates produced by the rolling tanks have been shown to be similar to those produced in the natural environment and therefore may be considered representative of naturally occurring aggregates.

Variations in aggregate abundance and size was observed in the *Eps* and *NC* tanks of both experiments. The macroaggregate abundance in *Eps* tanks varied from 9.8 to 60.9 cm^{-2} (experiment I) and 7 to 59 cm^{-2} (experiment II) (**Table 4.2**). Particles were also observed in the control tanks of both the experiments. It was clear that the autoclaved medium contained particles (mainly salts) at the start of each experiment (**Figs. 4.1a & 4.1b**). It is possible that the initial particle abundance might have influenced the overall aggregation by increasing the chances of particle collisions (Simon et al 2002). However, smaller aggregate size in the controls (*NC* tanks) suggests that initial abundance of inorganic particles alone could not produce macroaggregates. Exopolymers produced by microorganisms are known for their binding and gelling properties, which is essential for particle aggregation (Decho 1990, Herndl 1993, Hoagland et al 1993, Zhou et al 1998). This was evident from the production of larger sized particles in the *Eps* tanks supplemented with EPS derived from both bacteria and diatom.

The observed variations in particle abundance indicate that the production of aggregates was not a unilateral process and varied with time. An increase in

particle size with a concomitant decrease in particle abundance suggests that smaller aggregates were coagulating due to shear produced in the rolling tanks to form fewer but larger aggregates. This was evident from the data obtained for experimental tanks supplemented with bacterial and diatom EPS, especially after 20h (experiment I) and 28h (experiment II), respectively (**Fig. 4.2a & 4.2b**). During some samplings, aggregate counts increased with negligible change in the overall size of the aggregates. One reason for the observed variation could be the production of new particles. Similarly, disaggregation of particles might also contribute to the increase in particle abundance over time. Thus production of new particles combined with aggregation and disaggregation of particles appeared to be occurring during these experiments.

Aggregation experiments in laboratory and in natural environments have showed an increase in abundance of visible aggregates in the presence of polymers released by microorganisms (Reibesell 1991, Kiørboe and Hansen 1993, Passow et al 1994 and 1995, Stoderegger and Herndl 1998). However, all these observations were carried out using growing cultures of microorganisms in the laboratory or during phytoplankton blooms in the natural environments. In the present study, the influence of live cells was minimal. Therefore, an increase in particle abundance in the present study was not always possible and production of new particles was limited.

The presence of organic compounds like EPS help in increasing the chances of colliding particles to stick together and form larger particles (Herndl 1993,

Kiørboe and Hansen 1993). In the present study, experimental tanks containing only EPS in both the experiments (I & II) produced larger aggregates in first 20 to 46 h following incubation (**Fig. 4.2a & 4.2b**). Interestingly, similar aggregation rates were not observed when bacterial EPS was mixed with killed bacterial cells. Moreover, killed bacteria did not form any large sized macroaggregates in presence of diatom EPS. A similar variation in macroaggregate size was evident in the killed bacterial control tanks. The presence of killed bacterial cells was expected to serve as nuclei for macroaggregate production. Moreover, the rate of macroaggregate production is related directly to the stickiness of the colliding particles (Kiørboe et al 1990) and collision rate (which is a function of shear and particle density) (McCave 1984, O'Melia and Tiller 1993). However in the present study, the presence of killed bacterial cells appeared to suppress the aggregation process even in the presence of EPS. It is possible that killed bacterial cells might have released its intra-cellular content in to the medium, which might have had a suppressing effect on the aggregation of killed bacterial cell. Production of organic compounds that lower the particle stickiness has been reported in earlier studies (Decho 1990, Kiørboe and Hansen 1993). However, the exact cause of the suppression of macroaggregates in presence killed bacterial cells could not be ascertained in this study.

Differences in aggregation rates and size were observed in the *Eps* tanks between the two experiments. Single way ANOVA (**Table 4.4**) showed significant differences in the mean size and abundance of aggregates produced by EPS derived from different sources. These EPS differ in their

chemistry and binding properties (Kiørboe and Hansen 1993, Passow 2002) and therefore might influence the aggregation rate and macroaggregate size. One reason for the observed differences in aggregation rate, size and abundance could be the difference in the EPS chemistry. For example, *Marinobacter* EPS contained more of protein (44%) and uronic acid (17.6%) and lesser amounts of carbohydrates (17 %) and sulphates (2.7%) (**See Chapter 3**). In contrast, EPS of *S. costatum* had greater amounts of sulphates (21.5%) and relatively lesser amounts of proteins (3.6 %) and uronic acid (4.6 %) (**Table 4.2a**). Although both EPS were heteropolysaccharides, diatom EPS had greater abundance of different monosaccharides compared to bacterial EPS.

High molecular weight compounds like EPS released by microorganisms generally contain uronic acid, sulphates and proteins. These compounds may impart an overall negative charge to the polymer, thereby help in binding and aggregation processes (Christensen 1989). Moreover, sulphates are known to impart gelling properties and may help in floc formations (Hoagland et al 1993, Zhou et al 1998). For example, Zhou et al (1998) reported the accumulation of sulphates along with deoxy sugars in the foams obtained during bubbling of seawater supplemented with algal polysaccharides, indicating that surface active polymers rich in deoxy sugars and sulphates can form particles abiotically. In the present study, larger sized flocs were produced in presence of diatom EPS that was relatively rich in sulphates whereas aggregates were formed at a slightly faster rate in tanks containing bacterial EPS. The differences in the uronic acid and sulphate content and

monosaccharide chemistry of the EPS of *Marinobacter* and *S. costatum* might therefore be responsible for the observed difference in the aggregation rates and size.

Organic particles like TEP are considered important for aggregate production (Kiørboe and Hansen 1993, Passow et al 1995, Stoderegger and Herndl 1998, Simon et al 2002). CSPs are also found abundant in natural environments though its role in particle aggregation is not clear. TEPs were virtually absent or very low in numbers at the start of each experiment (**Fig. 4.3a & 4.3b)** and generally was lower than those reported by Stoderegger and Herndl (1998) and Passow (2002). Moreover, in those experiments the TEP abundance increased with time. In contrast to the observation of TEP, CSP counts in the *Eps* and *NC* tanks varied considerably in the two experiments and were in the range of 10^3 particles ml^{-1} (**Figs. 4.4a & 4.4b**). Only field reports on the distribution of CSP are available (Long and Azam 1996, Berman and Viner-Mozzini 2001) and therefore direct comparison with these reports might not be appropriate due to differences in the experimental conditions. The low amount of TEP in the present study was intriguing since both bacterial and diatom EPS have fair amount of uronic acid, which can serve as a precursors for the production of TEP. However, this was not so as observed from the TEP abundance data (**Table 4.2**). One reason for the observed low numbers of TEP could be the quality of the EPS used. Leppard et al (1977) have reported that precursors of colloids like TEP are few nanometer in size. Moreover these precursors can pass through 8 kDa dialysis bags (Passow 2002). In the present study, the EPS used as a

precursor for TEP production had been dialysed through 8 kDa MW cut-off dialysis bags prior to the aggregation experiments. Therefore, it is possible that fair amount of TEP precursors were lost during dialysis, thereby responsible for the low TEP abundance in these experiments. However, macroaggregates were produced in spite of the low TEP abundance. This is also reflected in the poor correlations between TEP/CSP abundance with macroaggregate size. Thus, it appears that neither TEP nor CSP was necessary for the production of macroaggregates.

4.5 Conclusions

The presence of microbial EPS led to the production of macroaggregates up to the size of 22 to 35 mm^2. Such abiotic production of macroaggregates carries significance in the DOM-POM transformation at depths unaffected by surface turbulence. The aggregate size and abundance varied with the source of EPS, especially in the absence of killed bacteria. The observed differences in the abundance of macroaggregates, TEP, CSP and macroaggregate size might be related to the differences in polymer chemistry. Moreover, organic particles like TEP and CSP need not always be required for production of macroaggregates.

CHAPTER 5

Marinobacter EPS as a source of carbon to heterotrophs

Chapter 5

5.1 Introduction

Microbial (phytoplankton, bacteria, microzooplankton) exopolysaccharides (EPS) in the aquatic environments exists either in free form, constituting a part of dissolved organic matter (DOM) (Lignell 1990, Decho 1990, Heissenberger et al 1996) and/or is associated with particulate matter like marine snow, phytoplankton aggregates etc. (Simon et al 1990, Hoagland et al 1993, Passow et al 1994). A significant fraction of the organic carbon fixed by phytoplankton (up to 80%) and utilized by bacteria (45%) in the pelagic waters is released as EPS in to the surrounding waters as DOM (Stoderegger and Herndl 1998, Biddanda and Benner 1997). Many marine bacteria produce EPS as long as they are metabolically active and therefore act as a continuous source of DOM, especially in the pelagic waters (Heissenberger et al 1996). On the other hand, microorganisms growing on various surfaces including sediments, rocks and aggregates like marine snow are embedded in a matrix of EPS (Decho 2000). Apart from helping in attachment to surfaces (as in biofilms), the EPS matrix provides a microenvironment to the microorganisms wherein they recycle nutrients and other elements (Decho 1990, Azam 1998).

Microbial EPS released as DOM play an important role in marine trophic-web by forming an essential component of the microbial loop. Microbial EPS is actively degraded by bacterial heterotrophs to meet their carbon and energy

requirements (Azam et al 1983). The bacterial uptake of extracellular compounds produced by phytoplankton during their growth is well documented in both laboratory (Amon and Benner 1996, Fajon et al 1999, Anderson and Ducklow 2001, Goto et al 2001) and field studies (Lancelot and Billen 1984, Sell and Overbeck 1992, Thingstad and Billen 1994, Ducklow et al 1999, Weiss and Simon 1999, Descy et al 2002). The uptake of DOC by heterotrophs and its conversion to POC (as living cells) thus, forms an alternate pathway of channelizing organic carbon in the marine food-web, which can be utilized by bacterivores like nanoflagellates and other protists in the food-chain (Fenchel 1982).

Conversely, grazers that feed on phytoplankton aggregates and deposit feeders ingest EPS as they feed upon particles (Decho and Moriarty 1990) and microbial mats (Stein 1984, White and Findlay 1988). The ingestion of microbial cells by deposit feeders is not sufficient to meet their energy requirements (Cammen 1980, Moriarty 1982, Juniper 1987) and therefore EPS might serve as an alternate source of nutrition for these animals (Moriarty 1982, Decho and Lopez 1993). Although most deposit feeders and grazers ingest bacterial exopolymer, its assimilation may vary with animal and source of EPS (Harvey and Luoma 1984, Baird and Thistle 1986, Decho and Lopez 1993). Moreover, as microbial EPS are good adsorbents of metals, animals ingesting EPS coated particulate matter can accumulate metals in their tissues (Patrick and Loutit 1976, Bremer and Loutit 1986, Schlekat et al 1999, Selck et al 1999). On the other hand, EPS can adsorb DOM and make

it available to particle feeders, thereby providing additional organic carbon to the particle feeders (Luoma and Davis 1983).

There is limited information available on the possible role of bacterial EPS as an organic carbon source to higher organisms (Harvey and Luoma 1984, Decho and Moriarty 1990, Decho and Lopez 1993, Selck et al 1999). Moreover, most of the studies on bacterial mineralisation of microbial EPS have used phytoplankton exudates as DOM and there is very little information on the fate of bacterial EPS as a carbon source to bacterial heterotrophs in the pelagic waters (Stoderegger and Herndl 1998). Keeping in mind the significance of EPS in the marine food-web and the limited information on bacterial EPS in particular, experiments were carried out using high molecular weight radio labeled *Marinobacter* EPS to assess the importance of *Marinobacter* EPS as a carbon source to i) a benthic deposit cum filter feeding polychaete *Nereis (Hediste) diversicolor* (O. F. Müller 1776) and ii) by natural bacterioplankton population collected from Dona Paula Bay.

5.2 Materials and Methods

Bacterial culture

Pure culture of the bacterial isolate previously characterised and identified as *Marinobacter sp.* in Chapter 3 was grown in a 2 l flask containing 1 l of BSS medium (Appendix Ib) supplemented with 3% glucose, 0.05% peptone and

0.01% yeast extract for 72h at room temperature (28 ± 2°C) under static growth conditions.

Preparation of labelled EPS

After 72h of growth, 1ml of ^{14}C-glucose (100mCi/ml; Sp. Activity 11470 mCi/mmole) was added to the culture and further incubated for 24h. At the end of the incubation period, the cells were harvested and the EPS was extracted and concentrated as described in Chapter 3. The concentrated EPS was precipitated using ice-cold ethanol, the precipitates redissolved in small volume of deionized water and dialyzed using 8 kDa MW cut-off dialysis bags. The dialysate was then reprecipitated, lyophilized and stored at –20°C.

5.2.1 Bacterial EPS as a feed for Nereis (Hediste) diversicolor (O.F. Müller 1776)

Nereis (Hediste) diversicolor

N. diversicolor is a sediment dwelling polychaete belonging to the phylum Annelida, class Polychaeta, family Nereididae. The animal is reddish brown in color and the average adult length ranges from 60-120 mm (**Plate I**). The animal inhabits intertidal muddy substrata in J-shaped burrows and its distribution is restricted to temperate waters of northwest Europe. The animal displays both diverse filter feeding and deposit feeding depending upon the amount of phytoplankton in the overlying waters. The animal is omnivorous

feeding upon detritus, mud, benthic microbial mats, macroalgae, sediment associated microflora and suspended phytoplankton (Riisgård 1991, Vedel and Riisgård 1993, Costa et al 2000).

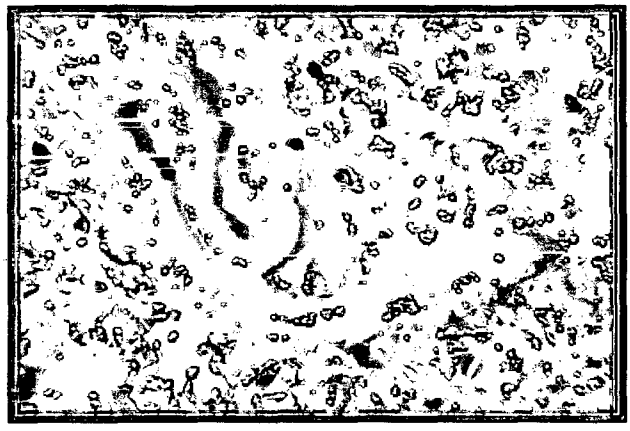

Plate I. Photograph of an adult *Nereis diversicolor*.

Animal collection

The polychaete *Nereis (Hediste) diversicolor* (O.F. Müller 1776) was collected from the intertidal sediments at Dornumerseil, northern German coast (**Fig. 5.1**). In order to collect the worms, sediment was collected using a shovel and rinsed through 1 mm mesh-sized sieve using seawater. The animals retained on the sieve were transferred into an aquarium and maintained in the laboratory. Before the feeding experiment, the animals were rinsed with filtered seawater (0.22 µm) to remove the adhered mucus coating and sediment and weighed.

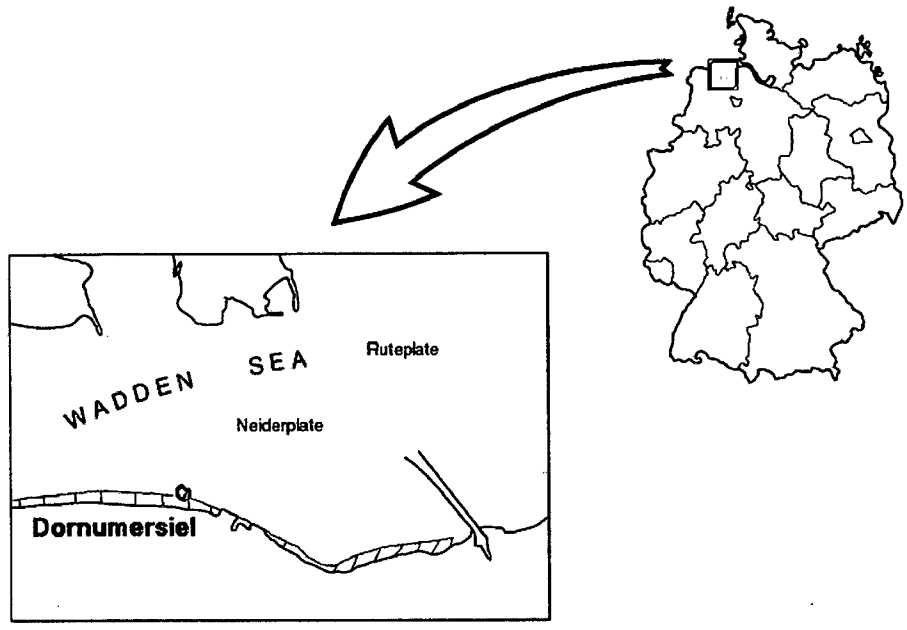

Fig. 5.1 Site chosen for the collection of the polychaete *N. diversicolor* for the feeding experiment.

Preparation of organic free sediment

Sediments used for the experiments were collected from the site of animal collection. Sediment was sieved to obtain an average grain size in the range of 250-500 µm. The sediments were then made organic–free following the method of Decho and Lopez (1993). Briefly, the sediments were soaked overnight in 30% hydrogen peroxide (H_2O_2) to oxidize the organic matter in the sediments. The sediment was then soaked in 0.5M NaOH solution containing 20 g l^{-1} $Na_4P_2O_7$. H_2O. This helped in removing the organic content of the sediment. After each treatment, sediment was washed in deionized water to remove the residual peroxide and $Na_4P_2O_7$. H_2O. The rinsed

sediment was dried at 100°C and ground with a pestle and mortar before using it in feeding experiment.

In order to prepare the feed, 50µl of the ^{14}C-labelled EPS solution (~811.2 ± 258.2 dpm) was made to 100µl in sterile distilled water and mixed with ~300 mg of sediment as prepared above in micro centrifuge tubes. The sediment-polymer mix was incubated for 2 h at room temperature and then transferred to an oven maintained at 50°C until the solution dried off completely. The sediments were then rinsed with 200 µl of autoclaved seawater and centrifuged at low speed (500 rpm) to remove loosely bound EPS. The supernatant was discarded and the sediment coated with labeled EPS was then used for feeding experiments.

Experimental setup

A schematic representation of the experimental setup is shown in **Fig. 5.2**. The test animals were fed on labelled feed for 24 h followed by 24 h of depuration. On the other hand, the negative control had live animals that were not exposed to the labelled feed. Formalin-killed animals that were exposed to labelled feed for 24 h were used as whole body controls.

For the feeding experiment, the test animals were transferred into sterile petri plates containing 20 ml of filtered seawater (0.22 µm) and 300 mg of sediment coated with ^{14}C-labelled EPS. Small quantities of dry powdered algae were also added to each petri-plate to facilitate the feeding. In all 7 live animals

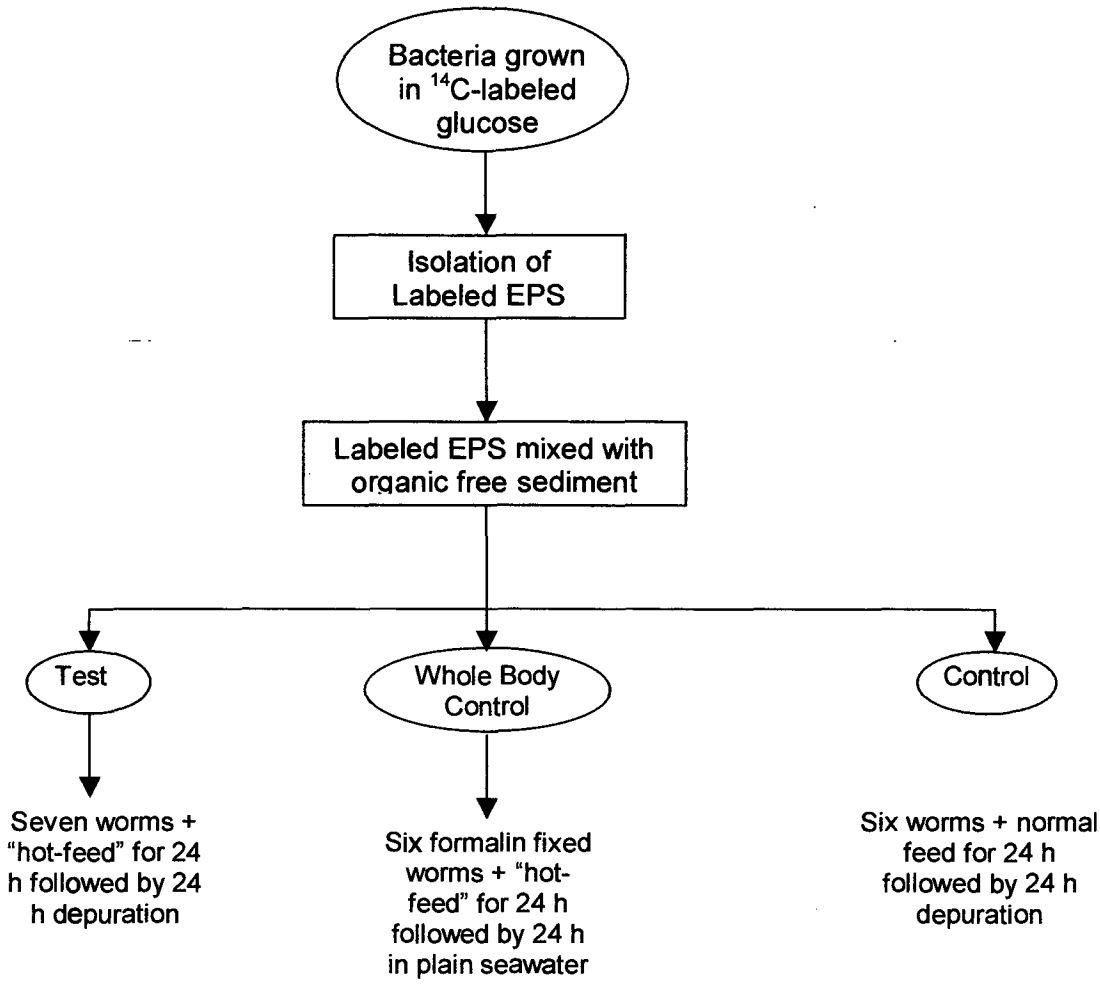

Fig. 5.2. A schematic representation of the experimental set-up used during the feeding experiment.

were used as test animals to estimate the assimilation of the labelled isotope by the animals. Similarly, another set of 6 formalin-killed worms was incubated in sterile petri-plates containing 20 ml filtered seawater (0.22 µm) and sediment (300 mg) coated with labelled EPS. This setup was used to estimate direct absorption of the labelled EPS on the body of the animals. For negative control experiment, 6 individual worms were taken in sterile petri

plates containing 20 ml filtered seawater (0.22µm) and fed with unlabelled feed. This experiment was used to estimate the background isotope counts in the body of the animals. These values were corrected in both the test and whole body control samples.

The animals were fed on sediments following the method described by Meziane and Thorin (1998). All the animals (tests and controls) were incubated at 15°C for 24h to allow feeding of sediment coated with labelled EPS and called hot feeding (HF). At the end of HF, animals from each petri-plate were removed and transferred to sterile petri plates containing only 20 ml of filtered seawater (0.22µm). The experimental and control animals were further incubated for another 24h under similar conditions to allow depuration.

Sampling and Analyses

At the end of feeding and depuration period, 1 ml of the seawater from the test and control petri plates was transferred to clean scintillation vials to estimate the total residual isotope in the seawater.

The amount of labelled EPS respired by the animal during both hot feeding and depuration was determined by phenylethylamine method. Briefly, the residual seawater from the test and control plates was transferred into screw-capped flasks using Pasteur pipettes. In each flask, papers soaked in phenyl ethylamine were suspended from a hook attached to the lid and the lids were tightly closed. One ml of concentrated glacial acetic acid was injected through a rubber septum in the lid to acidify the seawater. Care was taken not acidify

the phenylethylamine soaked papers. The flasks were kept overnight on a shaker to evolve carbon dioxide. The evolved CO_2 was trapped in the filter papers and the filter papers were then transferred into clean scintillation vials. The fecal matter accumulated in the petri-plates after the hot feeding and depuration experiment was transferred into scintillation vials using Pasteur pipettes. This fecal matter was then measured for the amount of labelled EPS excreted by the animals during the feeding experiment.

In order to estimate the amount of labelled EPS assimilated in the tissues, the test and whole body control animals were removed from the petri plates after the depuration period and rinsed in fresh 0.22 µm filtered seawater to remove the loosely adhered particles from the body surface. Both test and whole body control animals were then fixed in formalin and then transferred into tubes containing low quenching tissue solubilizer to dissolve the tissues and transferred to scintillation vials.

The negative control animals (without exposure to labelled feed) were also sampled and treated in a similar fashion. The seawater, respiration counts and whole body counts of the control were used as the blank and subtracted from the respective samples in both test and whole body controls.

All the samples of test and control animals including those for respiration, fecal matter, tissues and total residual polymer were analysed after the addition of scintillation cocktail. The isotopic counts were carried out on Wallac scintillation counter.

^{14}C-EPS in tissues after hot feeding

The amount of EPS retained by the tissues after 24 h of labelled EPS feeding was calculated as the sum of labelled EPS respired and retained in the tissues after depuration period. This is expressed as follows

^{14}C-EPS in tissues $_{\text{after feeding}}$ = [^{14}C-EPS respired + ^{14}C-EPS in tissues] $_{\text{after depuration}}$

% Assimilation efficiency

The amount of EPS assimilated (respired and in tissues) by the animals was estimated as

$$1 - \frac{[^{14}\text{C-fecal matter}]}{[^{14}\text{C-ingested}]} \times 100 = \%AE \quad \text{(Decho and Lopez 1993)}$$

and the total label ingested by the organism was estimated as

^{14}C-ingested= ^{14}C-respired + ^{14}C-in fecal matter + ^{14}C-in tissues.

5.2.2 Uptake of bacterial EPS by natural bacterioplankton

^{14}C-labeled bacterial EPS

The ^{14}C-labeled bacterial EPS used in the previous experiment was also used to study its heterotrophic mineralisation by natural bacterioplankton. A stock solution of bacterial EPS was prepared by dissolving a known amount of the

labeled EPS in 1 ml of sterile distilled water so as to obtain label concentration of 304080.7 dpm ml^{-1}.

Experimental setup

Aged seawater filtered through 0.22 µm filters and supplemented with nitrate and phosphate (as in f/2 medium), was redistributed equally (20 ml each) in 50 ml flasks. To each flask, 30 µl of the stock ^{14}C-labelled bacterial EPS (30480.7 dpm ml^{-1}) was added, which gave an initial isotope concentration of 940 dpm $flask^{-1}$. The flasks were then autoclaved and categorised into 2 sets having 10 flasks each. One set was used as experimental while the other was used as control.

Surface seawater collected from Dona Paula bay was filtered through 20 µm mesh and used as an inoculum. To each experimental flask, 1 ml of inoculum was added and the flasks were incubated in dark at room temperature (28 ± 2°C). The control flasks received 1 ml of the heat-killed inoculum.

Sampling

Duplicate flasks from both experimental and control sets were removed on day 0, 1, 3, 5, 9 and 11 following inoculation. Aliquots were drawn to estimate the total labelled substrate uptake, bacterial production and dissolved monosaccharides (MCHO). Moreover, the extra-cellular glucosidase activity (α-glc and β-glc) was estimated on the sampling days except on day 3.

Labelled EPS uptake and Bacterial production (Dual isotope measurements)

Both ^{14}C-EPS uptake and bacterial production was measured simultaneously during each sampling. In order to analyse the bacterial production, 10 ml of seawater was transferred from the test and control flasks into test tubes and 20 µl of thymidine (10 nM thymidine strength) was added to each test tubes. The test-tubes were incubated for 120 min at 28 ± 2°C in dark and shaken intermittently during the incubation period. At the end of the incubation period, the incorporation was terminated by adding 0.22 µm filtered formalin (4% final concentration). The samples were then filtered through 0.22µm cellulose nitrate filters pretreated with 5% trichloroacetic acid (TCA) (JGOFS protocol 1994). The particulate matter retained on the filters were rinsed thrice with ice-cold 5% TCA followed by three rinses of ice-cold 80% ethanol. The filters were transferred into scintillation vials and dissolved in ethyl acetate. One ml of the filtrate was transferred to scintillation vials to measure the residual isotope strength. Scintillation cocktail was then added to both particulate and residual samples and was measured simultaneously for both ^{14}C-EPS uptake and thymidine incorporation on a Wallac scintillation counter. The amount of ^{14}C-EPS incorporated was expressed as

$$^{14}\text{C-EPS uptake } (\mu g \text{ glucose } l^{-1} h^{-1}) = \frac{(Rs-Rb) \times A}{C \times t}$$ (Parsons et al 1984)

Wherein *Rs* and *Rb* are ^{14}C-counts (dpm) of sample and blank, *A* and *C* are initial concentration of substrate and isotope and *t* is the incubation time.

The details of the calculation of bacterial production are given in Chapter 2 b.

Extracellular glucosidase activity

In order to estimate the extracellular glucosidase activity, 5 ml of both test and control samples was transferred into clean vials. Ten µl of methyl-umbelliferyl-glucoside (MUF) substrate (10 nM final concentration) was added to the vials and the samples were incubated for 3 h. The fluorescence of methyl-umbelliferone released due to enzyme activity was then measured spectrofluorometrically at 365 $nm_{ex.}$ and 445 nm_{em} (Hoppe 1983). Methyl-umbelliferone was used as standard for the calibration of the method (See Chapter 2b).

Monosaccharide analyses

Seawater samples collected from both test and control flasks during the course of the experiment were filtered through 0.22 µm filter paper to remove bacterial cells and were stored at –20°C until analysis. A known volume was then analysed for monosaccharides (MCHO) content using 3-methyl 2-benzothiozolinone hydrazone hydrochloride (MBTH) method (Parsons et al 1984). In order to estimate dissolved MCHO concentrations, 1 ml filtered seawater sample was taken in a clean test-tube. To this sample, 0.05 ml potassium borohydride (KBH_4) was added and incubated in dark for 4 h during which the monosaccharides were reduced to form alditols. After the incubation period, equal volume of 0.7N HCl was added to stop the reduction

of sugars. The alditols were converted to formaldehyde by oxidizing for 10 mins with 0.1 ml periodic acid. The oxidation was terminated with 0.1 ml of sodium arsenite and acidified with 0.2 ml 2N HCl. To this mixture, 0.2 ml of MBTH was added to form complex with formaldehyde, which gave a colored solution in presence of ferric chloride. This solution was mixed with 1 ml of acetone and then measured spectrophotometrically at 635 nm. In the blanks, a mixture of equal volumes of periodic acid and sodium arsenite was added after the periodate oxidation step to prevent the production of formaldehyde from alditols. Thereafter, the rest of the method remained same. Glucose was used as the standard for calibration. All the analyses were carried out in duplicates.

5.2.3 Statistical Analyses

A simple correlation was performed to assess the relationship between the amounts of labeled carbon in different fractions (respiration, tissues, fecal matter and residual) during the feeding data. One-way ANOVA was used to estimate the significance in the differences of the isotope retained in the tissues of test and whole body controls. Similarly, correlation coefficients were also obtained for the mineralisation experiment to assess the inter-relationship between the uptake of labeled carbon and bacterial production against the variations in the MCHO and TDCHO concentrations and enzyme activity. The analyses were carried out using Statistica 5.0 software installed in the personal computer.

5.3 Results

5.3.1 EPS as carbon source for N. diversicolor

Adsorption of ^{14}C EPS to sediment

The total activity of ^{14}C-labeled polymer solution used to adsorb on the sediment was 16223 ± 5164 dpm ml^{-1}. The average concentration of the labeled carbon adsorbed to the sediments was 242 ± 60.3 dpm, which was 30 % of the labeled EPS added to 300 mg sediment (811.2 ± 258.2 dpm) (**Table 5.1**).

Individual worms weight (g)	Initial ^{14}C-label (dpm)	^{14}C-ingested (dpm)	^{14}C-residual (dpm)
297	266.4	95.45	170.9
628	162.8	72.75	90.0
656	231.9	27.85	204.0
845	234.8	77.07	157.7
850	297.4	91.45	205.9
1100	326.3	114.90	211.4
1165	174.0	64.37	109.6

Table 5.1: Ingestion of ^{14}C-labeled bacterial EPS coated on organic-free sediment by the polychaete *Nereis diversicolor*. The feeding was carried out for a period of 24 h.

Feeding of bacterial polymer by N. diversicolor

Feeding period- Feeding of the EPS coated sediment was found to be variable with the weight of the worms. At the end of the feeding period, the amount of ^{14}C-EPS ingested varied from 27.85 dpm to 114.9 dpm (**Table 5.1**). On the other hand, the residual amount of labeled EPS in the seawater (dissolved form) ranged from 90 to 248 dpm. On an average, the residual label was 185.4 ± 55.6 dpm

During the feeding period, certain amounts of ^{14}C-EPS ingested by the animals was excreted, respired or retained in the tissues of the animals. Except for one sample, the amount of labeled carbon respired ranged from undetectable to 61.1 dpm of $^{14}CO_2$ (**Table 5.2**). The labeled carbon in the fecal matter after 24 h of feeding ranged from 11.3 to 38.2 dpm (**Table 5.2**). The estimated labeled carbon retained in the tissues of the animal ranged from 5.45 to 99 dpm.

Depuration period- The depuration period was for 24 h during which no labeled carbon was excreted in the fecal matter. On the other hand, labeled carbon retained in the tissues after the feeding, was partly respired by some animals. Overall, the carbon respired ranged from undetectable to 99 dpm (**Table 5.3**). Thus up to 100% of the labeled carbon retained in the tissue after 24 h of feeding was respired as carbon dioxide during depuration.

Table 5.2. Amount of labeled EPS respired, excreted and retained in the tissues of the polychaete *Nereis diversicolor* after 24 h of feeding.

Number of animals	^{14}C-respired (dpm)	^{14}C-excreted (dpm)	^{14}C- in tissues (dpm)
1	61.10	26.50	7.85
2	nd	38.20	34.55
3	--	14.50	13.35
4	2.32	22.80	51.95
5	42.10	31.90	17.45
6	4.60	11.30	99.00
7	38.42	20.50	5.45

nd: not detected; --: Sample was lost.

Table 5.3. Amount of labeled EPS respired, excreted and retained in the tissues of the polychaete *Nereis diversicolor* after 24 h of depuration.

Number of animals	^{14}C-respired (dpm)	^{14}C-excreted (dpm)	^{14}C-tissues (test) (dpm)	^{14}C-tissues (control) (dpm)
1	nd	nd	7.85	nd
2	26.5	nd	8.05	nd
3	nd	nd	13.35	1.9
4	6.1	nd	45.85	nd
5	1.8	nd	16.45	nd
6	99	nd	nd	9
7	0.4	nd	5.15	

nd: not detected

The percent contribution of the different fractions of labeled carbon to the total ingested labeled carbon is shown in **Table 5.4.** At the end of the experiment, the total labeled carbon respired during the feeding and depuration period ranged from 10.9 to 90.1% **(Table 5.4)**. On an average, the amount of label respired was 48.6 ± 29.5% of the ingested labeled carbon. The percentage of labeled EPS excreted by the animals ranged from 9.8 to 52.2% of the

ingested EPS (**Table 5.4**). The average amount of labeled carbon excreted was 26.2 ± 10.8 dpm or 34.6 ± 15.3% of the total carbon ingested. The % of labeled carbon retained in the tissues of the animals at the end of the experiment ranged from undetectable levels to 59.5% (**Table 5.4**) of the labeled EPS ingested. On an average, this contributed to 28.7 ± 23.4% of the label ingested during the feeding period. From the table, it was clear that the percent of isotope retained by the animals was minimum compared to the amount respired or excreted. The absorption efficiency of the animals ranged from 47.9 to 90.2% (**Table 5.4**) and the mean absorption efficiency was 65.6 ± 15.1%. In comparison to the test animals, the amount of isotope retained by formalin-killed animals exposed by direct absorption into the tissues was detected in only 2 out of 6 animals (**Table 5.3**).

Number of animals	$\%^{14}$C-respired	$\%^{14}$C-excreted	$\%^{14}$C-retained	%AE
1	64	27.8	8.2	72.2
2	34.2	49.1	10.3	51.9
3	--	52.2	47.7	47.9
4	10.9	29.6	59.5	70.4
5	47.6	34.6	17.8	65.4
6	90.1	9.80	nd	90.2
7	60.1	32.0	8.03	68.0

Table 5.4: Percent of ^{14}C-labeled EPS respired, excreted, retained in tissues and absorption efficiency of the polychaete *Nereis diversicolor*.

Simple correlation coefficients were obtained for the total amount of labeled EPS ingested by the animals against other parameters including residual labeled carbon, concentration of labeled carbon respired, retained in tissues and excreted as fecal matter. There was no significant correlation of isotope ingestion and other parameters (**Table 5.5**). However, definite trends (r between –0.549 to 0.84) were evident between labeled-C respired and other parameters. One-way ANOVA of labeled EPS retained in the tissues of live test animals and whole body control animals showed moderately significant variance between the two sets (Fs= 11.3; $p<0.01$).

Parameters	Fecal matter	Residual	Tissue	Ingested
$^{14}CO_2$	-0.601	0.549	-0.740	0.840**
Fecal matter		-0.365	-0.162	0.088
Residual			0.002	0.319
Tissue				0.090

**: $p<0.01$

Table 5.5: Correlation matrix between various fractions of ^{14}C-labeled EPS and the total ingested ^{14}C-labeled EPS.

5.3.2 Heterotrophic uptake of bacterial EPS

Bacterial production and ^{14}-C uptake

The bacterial production in terms of carbon was maximal on day 1 and decreased with incubation time (**Fig. 5.3a**). The bacterial production rate declined from 1 ± 0.2 µg-C l^{-1} h^{-1} on day 1 to 0.24 ± 0.06 µg-C $l^{-1}h^{-1}$ by day 11.

On the other hand, bacterial production in the controls did not change during the experiment and ranged from 0.013 µg-C $l^{-1}h^{-1}$ on day 0 to 0.0069 µg-C $l^{-1}h^{-1}$ on day 11.

The variation in the amount of labeled carbon retained by the cells was similar to that of bacterial production (**Fig. 5.3b**). The uptake rates were maximal on day 1 (182.9± 3.8 µg-C l^{-1} h^{-1}) and decreased to a low on day 9 (31.3 µg-C l^{-1} h^{-1}) followed by a slight increase at the end (45.6 ± 1.2 µg-C l^{-1} h^{-1}). On the other hand, there were no observable differences in the uptake rates in controls.

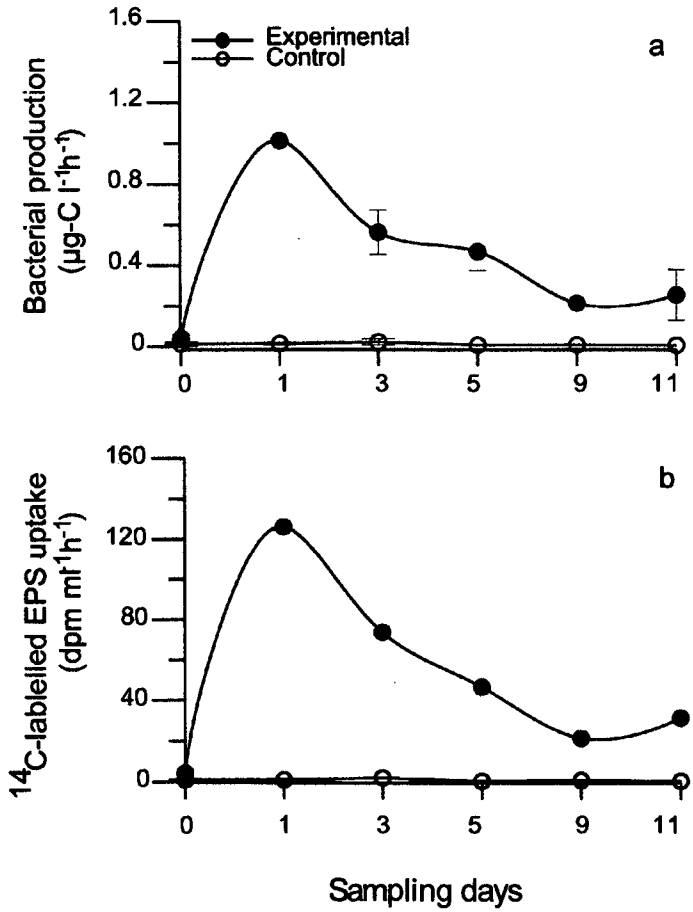

Fig. 5.3 Temporal variations of a) bacterial production and abundance and b) ^{14}C-EPS uptake in both experimental and control samples during the uptake of *Marinobacter* EPS by natural marine bacterioplankton. Each point is the mean of duplicate samples and error bar is the SD.

5.3.2b Extracellular glucosidase activity (α- and β-glucosidase)

During the mineralization experiment, β-glucosidase enzyme activity was always higher than that of α-glucosidase enzyme. However, the variation in enzyme activity was similar for both enzymes (**Fig. 5.4a**). On day 0, negligible activity was recorded in both α-glc (5.6 ± 0.4 μg-C l^{-1} h^{-1}) and β-glc (0 μg-C l^{-1} h^{-1}). However, a sharp increase in β-glc activity was registered (1324.8 ± 106.5 μg-C l^{-1} h^{-1}) in the next 24 h. Thereafter, the β-glc activity declined marginally to 907.3 ± 0.8 μg-C l^{-1} h^{-1} on day 9 followed by an increase to 1131.8 ± 45.3 μg-C l^{-1} h^{-1} on day 11. In contrast, α-glc activity did not increase much on day 1 (37.9 ± 3.1 μg-C l^{-1} h^{-1}) followed by an increase to 800.3 ± 5.3 μg-C l^{-1} h^{-1} on day 5. Thereafter, the variations in α-glc activity were similar to that of β-glc activity until the end of the experiment. The enzyme ratio plotted for the same period varied from 0.03 on day 1 to 0.69 on day 11 and showed variations similar to that of the enzyme activity (**Fig. 5.4b**).

Variation in MCHO concentrations

MCHO concentrations increased from 26.3 ± 9.8 μM on day 0 to 72.2 ± 1.5 μM on day 3 followed by a continuous decline until day 11 (11.1 μM) (**Fig. 5.5a**). The %MCHO used up by the microorganisms was 85% between day 3 and day 11. In comparison to the experimental samples, the control samples showed small-scale variations with a slight decline from day 0 (26.3 ± 9.8 μM) to day 5 (19.8 ± 1.1 μM) followed by an increase until day 11 (35.9 ± 1.9 μM).

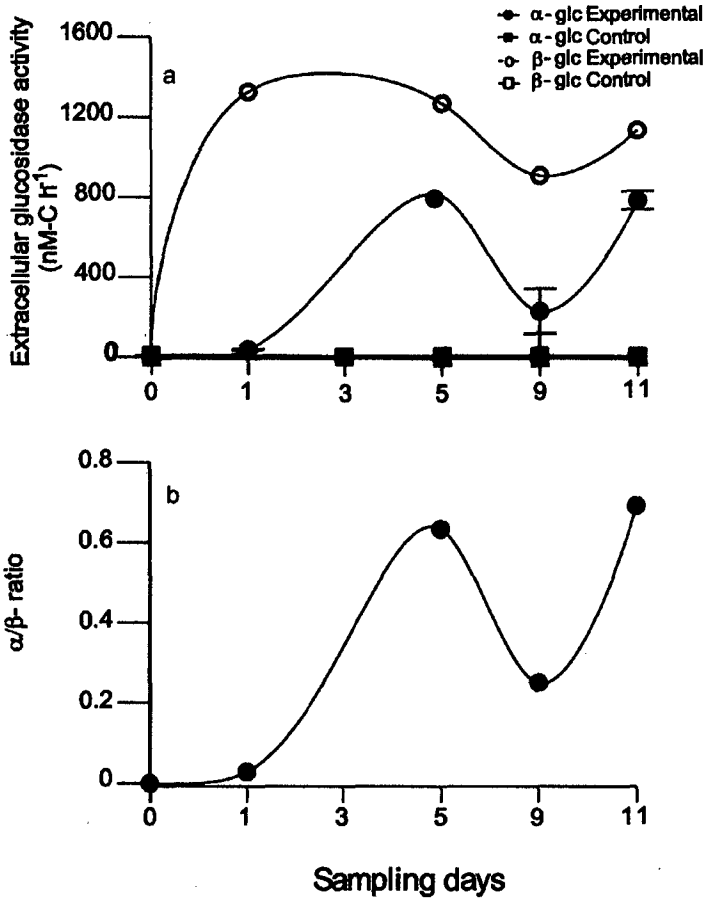

Fig. 5.4 Temporal variations in the activity of a) α-glc activity and β-glc activity in both test and control samples and b) α/β-ratio during the uptake of EPS by natural marine bacterioplankton. Each point is the mean of duplicate samples and error bar represents SD.

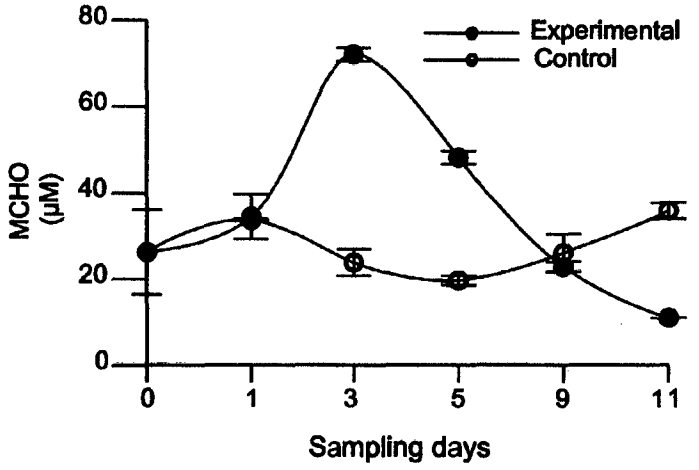

Fig. 5.5 Temporal variations of MCHO concentration in both test and control samples during the uptake of EPS by natural marine bacterioplankton. Each point is the mean of duplicate samples and error bar represents SD.

5.3.3 Statistical Analyses

The ^{14}C-incorporation rates and bacterial production were regressed against changes in the extracellular enzyme activity and MCHO concentrations (**Table 5.7**). Statistically significant linear relations were observed only between TBP and substrate uptake rates ($r=0.993$; $p<0.001$). Similarly, both TBP and substrate uptake rates showed positive trends with β-glc activity. In contrast, the bacterial production and susbstrate uptake rates had an inverse relation with MCHO concentrations (**Table 5.7**).

Parameters	Bacterial production	MCHO	β-glc activity	α-glc activity
^{14}C-uptake	0.993***	0.418	0.666	-0.182
α-glc activity	-0.127	0.042	0.514	-----
β-glc activity	0.707	0.255	-----	-----
MCHO	0.414	-----	-----	------

***: $p<0.001$.

Table 5.7 Correlation coefficients of various parameters monitored during the biodegradation of *Marionobacter* EPS by natural marine bacterioplankton.

5.4 Discussion

5.4.1 Bacterial EPS as a C-source for N. diversicolor (O.F. Müller 1776)

Bacterial EPS was ingested in varying amounts during the feeding experiment (**Table 5.1**). The amount of polymer ingested by the animals varied from 27.85 to 114.9 dpm (**Table 5.1**) during the entire feeding experiment ($\bar{X}$= 77.7 ± 27.6 dpm). This was only 32.6% of the label fed to the animals. It was apparent that the animals did not ingest most of the labeled carbon during the feeding period. The low amount of labeled carbon ingested in this experiment was in contrast to those reported for other sediment dwelling animals. Some polychaetes like *Streblopsio benedectii* (Decho and Lopez 1993) and sediment dwelling amphipods *Leptocheirus plumulosus* (Schlekat et al 1999) have been shown to ingest microbial EPS within hours of incubation. In contrast, filter feeders like *M. balthica* delayed the uptake of bacterial EPS by more than 12 h (Harvey and Luoma 1984). Various reasons including the amount of labeled carbon adsorbed by the sediment, the quality of the polymer (Decho and Lopez 1993), sediment characteristics and the feeding behavior of the animals might be responsible for the poor ingestion of the polymer.

Organic free sediment has been regularly used as a coating surface to study feeding behavior of deposit-feeding animals (Decho and Lopez 1993, Schlekat et al 1999). Since the sediment used in this study was obtained from the animal collection site (**Fig. 5.1**), characteristics of the sediment should not

have influenced the ingestion of the polymer. However, the amount of EPS adsorbed onto the sediment surface showed considerable variations. The coefficient of variation of mean EPS adsorbed (242 ± 60.3 dpm) on to the sediment was 25%, which was much higher than those recorded by Decho and Moriarty (1990). Unlike the bacterium-sized beads (< 2 µm) used by the Decho and Moriarty (1990), the sediment used in the present study did not receive any pretreatment prior to adsorption of the polymer. The beads used by Decho and Moriarty (1990) had larger surface area to volume ratio compared to sediment used in this study. The higher ratio and the pretreatment of the beads ensured better adsorption of EPS per unit weight of the beads. Despite the differences in the initial EPS adsorbed on to the sediment, there was not much of influence in the amount of EPS ingested by the animals (**Table 5.1**). Thus, the polymer chemistry or the feeding behaviour or both could have played a role in the poor ingestion of the EPS.

The %AE is a measure of the efficiency of an animal to absorb the food into its body, which then might be respired and/or retained in the tissues. Although the amount of EPS ingested by the animals was less, the absorption efficiency (%AE) was relatively high. The %AE of the animals ranged from 47.9 to 90.2% and on an average was 65.6% (**Table 5.4**). This was similar to those reported for another sediment dwelling polychaete *Streblopsio benedectii* fed on slime EPS derived from marine pseudomonads (Decho and Lopez 1993). The assimilation efficiency exhibited by *N. diversicolor* in this study however cannot be generalized for other feeders and EPS derived from other bacteria. Bacterial EPS has been used to study its significance for

benthic feeders including bivalves (Harvey and Luoma 1984), holothurians (Baird and Thistle 1986), copepods (Decho and Moriarty 1990), polychaetes (Decho and Lopez 1993) and amphipods (Schlekat et al 1999). However, EPS assimilation by the animals has varied considerably with both source of EPS and animal feeding on it. For example, EPS isolated from a marine pseudomonad bacterium was assimilated more efficiently in the tissues of holothurians (Baird and Thistle 1986) compared to that of bivalves (Harvey and Luoma 1984). Likewise, organisms like harpacticoid copepods could assimilate up to 80-85% of the ingested bacterial EPS (Decho and Moriarty 1990). From the earlier studies and the present set of data it is clear that the digestion and assimilation of microbial EPS was variable in different organisms.

Although the %AE was high, a major fraction of the polymer ingested was respired as carbon dioxide (51%) and only 28.7% was retained in the tissues (**Table 5.4**). However, our result was in contrast to the observations of Decho and Moriarty (1990) who reported only 14.6% respiration loss of the bacterial EPS by copepods. Moreover, the label respired by the animals in the present study had a positive relation with amount of the polymer ingested but inverse to the amount retained in the tissues or excreted out (**Table 5.6**). It is evident that the animals obtained measurable nutrition and energy from the ingested EPS.

The amount of labeled carbon retained in the tissues after depuration indicates the incorporation of the label into the structural components of the

tissue. The amount of label retained by the tissue might be due to ingestion and/or direct adsorption of the label from the DOM or both. One-way ANOVA carried out between the whole body controls and the whole body test samples showed significant differences in the variances (Fs= 11.3, $p< 0.01$) suggesting that direct adsorption of the labeled polymer as DOM by the tissues should not have influenced the live animal data. This was in contrast to those reported by Decho and Lopez (1993) wherein both the live and killed animals retained more or less similar amount of label during the course of the experiment.

The assimilation efficiency is influenced by the quality of the EPS (Decho and Lopez 1993), gut passage time and an efficient enzymatic system capable of digesting the EPS (Harvey and Luoma 1984, Baird and Thistle 1986). The composition of EPS appears to play an important role in the degradation and the assimilation of EPS as observed in some studies. For example, capsular EPS of *Pseudomonas* was less preferred over the slime form by the polychaete *Streblopsio benedectii* (Decho and Lopez 1993). Slime EPS is rich in polysaccharides that are considered to be more labile compared to protein rich capsular EPS (Decho 1990). The bacterial polymer in the present study had low carbohydrate and high protein concentrations (see Chapter 3), which was also reflected in its low C:N ratio. Despite the low amounts of the polymer ingested by *N. diversicolor*, the animal showed the capability to digest the EPS and assimilate a major portion of the polymer. Similar observations have been made in a polychaete *Arenicola marina* that was able to digest the capsules of both gram-positive and gram-negative bacteria (Plante 2000).

This suggests that apart from the chemistry of the EPS, the nature of digestive enzymes produced by the feeding animal and the gut-associated microbial flora also influence the digestion of EPS and its subsequent assimilation in the tissues (Baird and Thistle 1986, Decho 1990).

The availability of bacterial EPS as a carbon source can have several implications in the benthic food chain. For example, bacterial capsules are made of highly complex, less hydrated, HMW fibrillar polysaccharides (Heissenberger et al 1996) that may contain various radicals like carboxylates, pyruvates, sulphates etc. (Decho 1990) and complex organic compounds like proteins (Wingender et al 1999, Sutherland 2001). Such compounds impart a negative charge to the EPS and help in adsorption of organic matter like DOM and metal ions (Geesey et al 1988, Decho 1990), thereby make it available to the higher trophic level (Luoma and Davis 1983). On the other hand, many bacteria may use the dissolved organic carbon and produce EPS (Stoderegger and Herndl 1998). This EPS is adsorbed by the sediments (Decho 2000) and the ingestion of EPS coated sediments by invertebrates suggests that a large fraction of the carbon fixed in the marine environment can enter the food-chain through the production of EPS.

5.4.2 Heterotrophic uptake of labeled bacterial EPS by natural bacterioplankton

The breakdown and subsequent uptake of labeled bacterial EPS by natural bacterioplankton was estimated as the amount of labeled carbon incorporated in the bacterial cells over time. The labeled *Marinobacter* EPS was actively incorporated by natural microflora from Dona Paula Bay during the first 24 h of incubation (**Fig. 5.1b**). This was accompanied by a similar increase in bacterial production rate (**Fig. 5.1a**) and β-glc activity (**Fig. 5.4a**). Since the polymer used was > 8 kDa, its incorporation into the cells will require enzymes that can breakdown the EPS to smaller size before getting incorporated into the cells. The concomitant increase in ^{14}C-incorporation and β-glc activity in the first 24 h suggests a close link between the ^{14}C-uptake and enzymatic breakdown of the polymer. The rapid breakdown and incorporation of extracellular organic compounds like EPS by natural bacterioplankton has been reported using phytoplankton (Chrost 1990 & 1991). Moreover, such an incorporation of ^{14}C-labeled EPS was tightly coupled with bacterial production indicating the influence of bacterial EPS in regulating bacterial production. This was also supported by a significant correlation ($r= 0.99$; $p<0.001$) between bacterial production and ^{14}C-labled uptake observed during the study period.

The incorporation of bacterial EPS observed in this study was in contrast to those reported by Stoderegger and Herndl (1998). According to these authors, bacterial capsular material were not degraded efficiently even in the

presence of nutrients like phosphates and nitrates. On the contrary, EPS isolated from *Azotobacter vinelandii* were actively degraded and mineralized by limnic microbes (Weaver and Hicks 1995). From these studies and the present data, its clear that bacterial EPS are not always a good source of carbon for heterotrophs. Several reasons including differences in the chemistry of the EPS, composition of the microbial population and the nature of enzymes produced by the bacteria can be responsible for the varied response. For example, EPS rich in structural components like proteins to stabilize its overall structure (Decho 1990, Hoagland 1993) might help the EPS to resist enzymatic degradation in the natural environments. On the other hand, dissolved EPS produced in the natural environments might be rich in nitrogen with dissolved amino acids and thus are actively degraded by marine bacteria (Decho 1990). The carbohydrates of the *Marinobacter* EPS used in the present study was rich in glucose (79 mol%) but also had considerable amounts of proteins (See Table 3.4, Chapter 3). It is possible that the higher amount of glucose and proteins present might induce microbial uptake of the EPS.

A steady decline in bacterial production and incorporation of bacterial EPS was observed after day 1 of the incubation. The subsequent decline in labeled carbon uptake and bacterial production indicates the depletion of labeled EPS concentrations in the medium. However, there was no concomitant decline in the enzyme activity indicating that labeled EPS was not the only carbon source used by the natural bacterioplankton population. Although aged seawater was used to carry out this experiment, the DOC present in the

seawater might also serve as an additional carbon source for the bacterioplankton. Alternately, extracellular organic compounds released by bacteria during its growth might also serve as an alternate source of organic carbon for other heterotrophs.

The enzymatic breakdown of polymers like EPS yields monomers that are subsequently utilized by growing heterotrophs. In the present study, MCHO concentrations did not change much in the first 24 h of incubation (**Fig. 5.5)** despite higher bacterial production and enzyme activity. On the other hand, the decline in the MCHO concentrations coincided with reduced bacterial production. This suggests that MCHO released by enzymatic hydrolysis of EPS was immediately utilized for bacterial production. The removal of low molecular weight carbohydrates has been reported in both laboratory and field studies (Sell and Overbeck, 1992, Agis et al 1998, Janse et al 1999). Low molecular weight compounds are easily assimilated in to the cell and hence are more preferred for heterotrophic growth. Monomers like glucose have been identified as a major carbon source for many marine bacteria (Carlson and Ducklow 1996).

The production of specific enzymes is used to assess the quality and the chemistry of organic matter (Chrost 1989, Herndl 1995). The β-glc activity on day 1 was greater than the α-glc activity by an order of 10 and always remained greater than α-glc activity. Moreover, the enzyme ratios (α/β) during the experiment were always less than 1. This indicates that β-glycosidic linkages were more abundant in the bacterial polymer. According to some

reports, higher β-glc activity is generally associated with less degradable organic matter (Müller-Niklas et al 1994). Chrost (1989) reported higher β-glc activity in natural waters when detrital organic matter (senescent algal cells) accumulated in the water column. On the other hand, the delay in increased α-glc activity might be in response to the production of easily degradable lower molecular weight compounds released during the breakdown of EPS. Moreover, the production of extracellular compounds by the growing heterotrophs might also induce the production of α-glc enzymes.

The active incorporation of labeled bacterial EPS by natural bacterioplankton may have ecological implications. In oligotrophic waters, the organic carbon demand generally exceeds the supply. Under such conditions, the amount of organic carbon transformed through the microbial loop gains significance (Azam 1998). Since bacterial EPS can contribute considerably to the total organic carbon pool (Stoderegger and Herndl 1998), a rapid enzymatic breakdown and incorporation of bacterial EPS by the natural bacterioplankton can help in meeting the carbon demand of heterotrophs, which in turn sustain the higher trophic levels.

5.5 Conclusions

Bacterial EPS as a carbon source had variable responses from benthic feeders and microbial heterotrophs. Although the bacterial EPS was not ingested in large amounts, a major portion (65.5%) of the ingested polymer was assimilated by the polychaete *H. diversicolor* grown and retained about

30% of labeled EPS in tissues. The ingestion and assimilation of EPS might also have significance in the accumulation of metals adsorbed by the EPS in the tissues of the deposit feeders. On the other hand, heterotrophic incorporation of bacterial EPS by natural bacterioplankton decreased with time. This coincided with decrease in MCHO concentration over time, suggesting that bacterial EPS were broken down to smaller compounds and then used to support bacterial growth and production. Thus, bacterial EPS can serve as an alternate source of organic carbon to microbial heterotrophs.

CHAPTER 6

Metal binding properties of the *Marinobacter* EPS

Chapter 6

6.1 Introduction

Metals like Pb, Cd, Sr, Ag, Sn, Hg etc are non-essential metals, and the presence of these metals above certain concentrations in the aquatic environment can be toxic to organisms (Venugopal and Luckey 1978, Gadd 1992). The toxic metals in the dissolved state are taken up by growing cells by liganding with the intracellular organic molecules (Roanne and Pepper 2000). However, certain microorganisms can tolerate the presence of toxic metals and also accumulate and/or precipitate these metals when exposed to sub-lethal concentrations. Resistance to metals basically involves immobilization of dissolved metals in the natural environment, thereby reducing its bioavailability (Roanne and Pepper 2000). The resistance to toxic metals can be either by non-specific & constitutive (slime layer production, siderophores and biosurfactants), non-specific & inducible (production of metallothioneins and peptides, metal efflux mechanisms) or specific and inducible (metal transformations like methylation) mechanisms. The accumulation of heavy metals occur either by extracellular adsorption (Brown and Lester 1982, Rudd et al 1984, Gaddie and Sutherland 1990, Loaëc et al 1997 and 1998, Slaveykova and Wilkinson 2002), cell surface adsorption (Jalali and Baldwin 2000) or metal leaching from metal sulfides (Sand and Gehrke 1997). The extracellular adsorption of metal ions by EPS-producing microorganisms is economical and efficient and is more preferred in bioremediation of toxic metals.

Bacteria produce extracellular compounds like exopolysaccharides (EPS). These polymers are high molecular weight compounds having charged functional groups that have sorptive properties (Decho 1990). Since EPS exists in free (as DOM) and cell-associated (as in biofilms) forms, it ideally serves as a ligand source for relatively easy and efficient removal of heavy metals from natural environments (Decho 1990, Chen et al 1995). For example, various genera of bacteria like *Staphylococcus, Azotobacter and Micrococcus* produce EPS that can bind heavy metals (Roanne and Pepper 2000). Similarly, sulphate-reducing bacteria also bind heavy metals using their EPS (Bridge et al 1999). Biosorption of metals by EPS is also reported for other organisms like fungi (Guibal et al 1992, Mullen et al 1992, Tobin and Roux 1998), yeast (Wakatsuki et al 1979, Donmez and Aksu 2001) and algae (Kaplan 1988, Mehta et al 2002).

In the marine environment, the binding of metals by microbial EPS can have far-reaching implications in the biogeochemical cycling of metals, removal of pollutants from the water column and the bioaccumulation of pollutants in the marine organisms. Exopolysaccharides produced by phytoplankton and bacteria form an intrinsic component of both the DOM and marine aggregates like marine snow (Decho 1990). EPS in either form adsorb trace metals and are involved in the cycling of metals in the pelagic environment (Tye et al 1996, Buffle et al 1998). Since these organic polymers have greater binding capacity for metals than any other known mineral sorbent (Quigley et al 2002), they cause multiple complexation of metal ions and remove them from the overlying waters and transport it into the sediments. The ubiquitous

presence of a class of transparent exopolymeric particles (TEP) in the aquatic environment has been implicated in the transport of trace elements like thorium (Niven et al 1995). Similarly, EPS in marine aggregates like marine snow and large phytoplankton aggregates (Alldredge and Silver 1988, Herndl 1993, Decho and Herndl 1995) can also cause metals to condensate and get transported to the bottom sediments. Biofilm EPS found on sediments, intertidal mudflats and other submerged surfaces are known to adsorb heavy metals (Nelson et al 1999, Decho 2000, Hunt et al 2001). In the deep-sea, bacterial mats growing in the vicinity of hydrothermal vents tend to accumulate minerals in their capsular material (Cowen and Silver 1984, Cowen et al 1986). Moreover, EPS isolated from bacteria inhabiting near the vents show high affinities for metals like Pb, Ni, Cd, Zn, Cu etc. (Brown and Lester 1982 & 1984, Rudd et al 1984, Loaëc et al 1997 & 1998, Dong et al 2000). Such properties can be exploited for bioremediation of pollutants in the environment.

Microorganisms and biofilms accumulate metals in the marine environments and play and important role in the transfer of toxic metals to higher animals in the marine trophic food-web (Roanne and Pepper 2000). For example, many filter feeding and deposit feeding organisms are reported to accumulate toxic metals (Harvey and Luoma 1984 & 1985, Decho and Lopez 1993, Schlekat et al 1999).

Keeping in mind the usefulness of bacterial EPS in the bioremediation of metals, its ecological significance and their role in the transfer of these metals

into the marine food-web, a study was carried out to assess Cu and Pb binding by *Marinobacter* EPS. Effect of factors such as pH and sodium chloride on the binding EPS these metals was evaluated.

6.2 Materials and Methods

6.2.1 Production and isolation of Marinobacter EPS

Pure culture of *Marinobacter* sp. was grown in 2 l flasks containing 1 l of the optimized BSS medium adjusted to pH 7.6. The culture was grown at 30°C ± 0.5°C over a period of 14 days. At the end of the incubation period, the cells were harvested and the supernatant was collected. The cell pellet was treated with 10mM EDTA as described in Chapter 3 to extract cell-bound EPS. The EDTA-extracted EPS was pooled with the supernatant EPS solution and filtered through 0.22 µm filters, concentrated using a rotavapor at 30°C to less than 50 ml. This bacterial EPS solution was then precipitated overnight using ice-cold absolute ethanol at 4°C overnight (Bhosle et al 1995), supernatant was decanted and the precipitates were dissolved in small volumes of distilled water and dialysed in 8 kDa MW cut-off dialysis bags to remove salts. The high molecular weight fraction EPS retained in the dialysis bag was then reprecipitated, lyophilised and stored at –20°C until further analyses.

Prior to each metal-binding experiment, a known amount of bacterial EPS was dissolved in known volume of deionized water to give a final concentration of 5.6 ± 0.34 mM-C. This solution was used directly for metal binding assay.

6.2.2 Preparation of metal solutions

Stock solutions having 40 mM copper and 100 mM lead were prepared by dissolving copper sulphate ($CuSO_4.5H_2O$) and lead nitrate ($Pb(NO_3)_2$) in deionized water, respectively. The stocks were stored at 4°C and working solutions of adequate concentrations were obtained after appropriate dilution in deionized water prior to each experiment.

6.2.3 Washing of dialysis bags for metal-binding assay

Prior to each assay, 8 kDa molecular weight cut-off fresh dialysis bags (Spectrapor membrane tubings) were first washed with 10 mM EDTA in 1% Na_2CO_3 at 100°C for 30 mins followed by a wash in distilled water at 100°C for another 60 mins as described by the manufacturers.

6.2.4 Influence of incubation time on metal-binding by EPS

In order to assess the optimal time required for maximum binding of dissolved copper and lead bound by bacterial EPS, clean dialysis bags containing 2 ml of the bacterial EPS solution (5.6 mM-C concentration) were suspended in wide-mouthed PVC bottles having solution of copper (40 mM) or lead (10 mM) and incubated on a shaker at 220 rpm at room temperature (28 ± 2°C). The dialysis bags were retrieved after 40, 60, 70, 80, 120, 160, 200 and 240 mins of incubation and transferred to another polyvinyl container having distilled water and dialyzed overnight to remove any loosely bound metal ions. The

metal complexed polymer solution was then transferred to clean plastic vials and stored at –20°C prior to analysis.

6.2.5 Effect of initial metal ion concentration

In order to assess the binding capacity of the bacterial polymer, saturation experiments were carried out using increasing concentrations of both lead and copper. Bacterial EPS solution was taken in dialysis bags and suspended in solutions of varying concentrations of copper (4, 5, 6.4, 8, 10 & 13 mg l^{-1}) and lead (1.3, 1.9, 2.5, 4, 5, 6.4, 8, 10 & 13 mg l^{-1}). The dialysis bags were first incubated for 120 mins in the metal solutions, then transferred into distilled water and dialyzed overnight to remove the unbound metal ions.

6.2.6 Effect of pH on metal-binding by EPS

Solutions of lead (10 mg l^{-1}) and copper (4 mg l^{-1}) were prepared in deionized water and the required pH was adjusted using 1 N NaOH. The binding capacity was assessed over a pH range of 4.5, 5, 5.5, 6, 6.5, 7 and 7.5. Dialysis bags containing the bacterial EPS solution were suspended in the metal solutions of varying pH for 120 mins. After incubation, the dialysis bags were dialyzed to remove unbound metal ions as described previously.

6.2.7 Influence of sodium chloride on metal-binding by EPS

The influence of sodium chloride on the binding capacity of bacterial EPS was also assessed. A known volume of stock solution of copper (40 mg l^{-1}) and lead (100 mg l^{-1}) were diluted in open-mouthed PVC bottles containing increasing concentrations of sodium chloride (0%, 1%, 1.5%, 2%, 2.5%, 3% & 3.5%) solution to obtain a final concentration of 4 mg l^{-1} copper and 10 mg l^{-1} lead. The pH of the metal solutions was adjusted to 7 using 1 N NaOH. Dialysis bags containing 2 ml of bacterial EPS was allowed to complex with the metal ions for 120 mins. After incubation, the dialysis bags were dialyzed overnight against deionized water to remove unbound metal ions.

In all the assays described above, bacterial EPS replaced with distilled water and treated in similar fashion was used as blanks. Similarly, dialysis bags containing bacterial EPS solutions incubated in distilled water were treated in the same fashion and used as controls. All the analyses were carried out in duplicate.

The concentration of copper and lead in the samples, control and blank was measured using a double beam atomic absorption spectrophotometer (GBC-902 model; GBC Scientific Inc., USA). Calibration of instrument was carried out by analyzing the absorption of increasing concentrations of lead nitrate and copper sulphate solutions.

6.2.8 Estimation of maximum binding ability (MBA)

The maximum binding ability (MBA) of the polymer for a given metal can be estimated from the langmuir plots drawn between the ratio of free ions to bound ions (mg^{-1}) against the available free ions using the equation

$c/x = 1/k_1k_c + c/k_1$; (Mittleman and Geesey 1985)

where c is the free ion concentration, x is the bound ion concentration, k_1 is maximum binding ability (MBA) and k_c is the conditional stability constant. This equation is also used to estimate the distribution coefficient (slope of the plot) of the metals.

6.3 Results

6.3.1 Influence of incubation time on metal-binding by EPS

The amount of free copper and lead ions complexed by the polymer over time is shown in **Fig. 6.1a & 6.1b**. Concentrations of copper ions adsorbed by the EPS increased with time and reached a highest value of 434.4 ± 6 mg g^{-1} EPS after 120 min following incubation. A similar adsorption pattern was observed for lead with the maximal adsorption (69.9 ± 6.8 mg g^{-1} EPS) following 120 min of incubation.

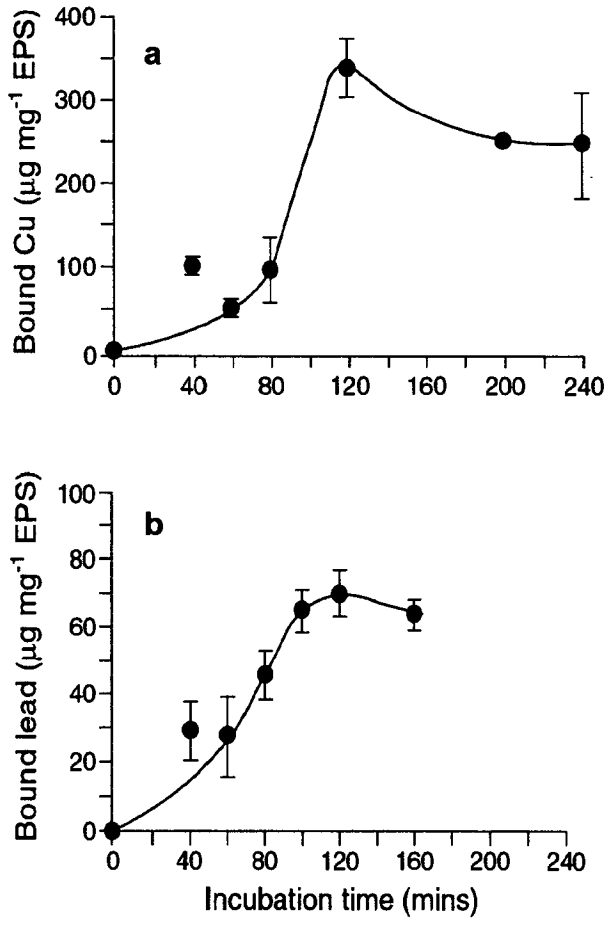

Fig. 6.1 The changes in the amount of (a) copper and (b) lead bound by EPS produced by *Marinobacter* sp.

6.3.2 Effect of initial metal ion concentration on metal-binding by EPS

The amount of copper and lead adsorbed by the EPS with increasing concentration of copper and lead is shown as equilibrium isotherms in Fig. **6.2 a**. From the figure, it is clear that amount of lead adsorbed by the EPS was lesser than copper and reached state of equilibrium (39.3 ± 1.9 mg g^{-1} EPS) when the initial lead concentration was 5 mg l^{-1}. Thereafter, the amount of lead adsorbed did not change much with increasing lead concentration. On

the other hand, the amount of copper adsorbed increased with increasing copper concentrations and varied from 162.9 ± 6.1 mg g^{-1} EPS at 4 mg l^{-1} copper concentration to 189 mg g^{-1} EPS at 10 mg l^{-1} copper concentration.

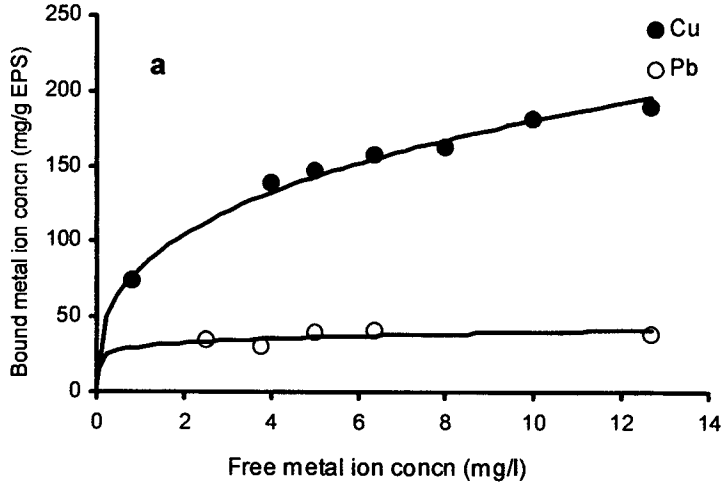

Fig. 6.2a Equilibrium sorption isotherms of lead and copper after binding by EPS of *Marinobacter* sp. The experiment was carried out at 220 rpm.

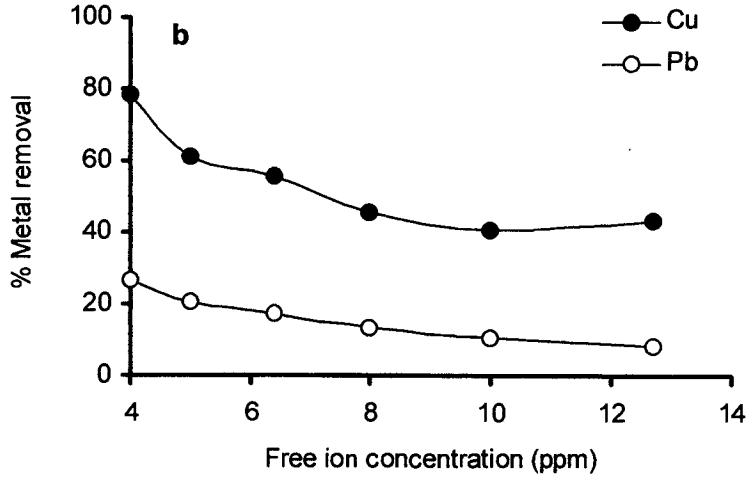

Fig. 6.2b Amount of free metal ions removed over increasing concentrations of lead and copper by EPS of *Marinobacter* sp.

Notable differences were observed in the amount of metal adsorbed when expressed as % metal removed from the solution (**Fig. 6.2b**). For example, at an initial free metal concentration of 4 mg l^{-1}, the amount of copper removed (78.2%) was 3 folds greater than the amount of lead (26.7%) removed by the EPS. Moreover, the amount of metal ion removed decreased with increasing concentration of metal ion concentrations (**Fig. 6.2b**).

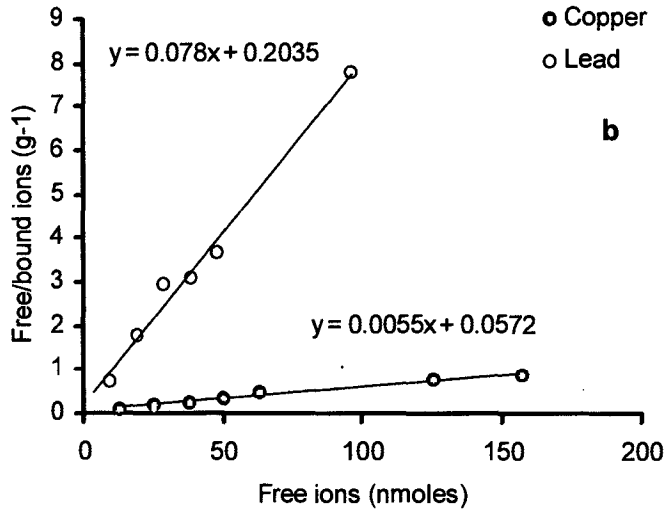

Fig. 6.3 Langmuir's linear isotherms for copper and lead bound to crude exopolymer.

A langmuir's linear relation was plotted between the ratio of free:bound metal ions and initial free ion concentration (**Fig. 6.3).** For better comparison with other worker's data, we recalculated the bound copper and lead concentrations obtained from the equilibrium isotherms (**Fig. 6.**2a) in nmols mg^{-1} carbohydrate and used it to plot the langmuir's isotherms. Using the equation of the langmuir's isotherm, maximum binding ability was calculated

for both metals. The maximum binding ability for copper and lead was 181.8 nmol mg^{-1} carbohydrate and 12.8 nmol mg^{-1} carbohydrate, respectively.

6.3.3 Effect of pH on metal-binding by EPS

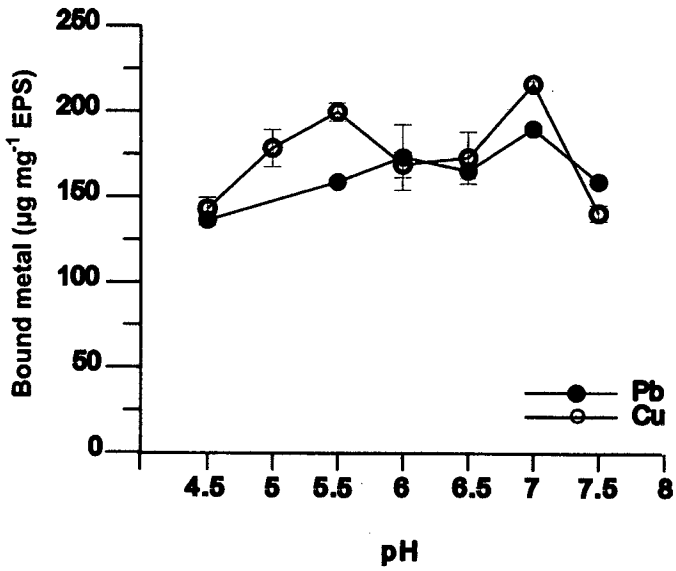

Fig. 6.4 Effect o pH on the amount of copper and lead bound by *Marinobacter* EPS.

Both copper and lead were adsorbed more in near neutral pH than in acidic pH. The amount of copper adsorbed by the EPS showed an increase from 163 ± 6.1 mg g^{-1} EPS at pH 4.5 to 192.7 mg g^{-1} EPS at pH 7 (**Fig. 6.4**). Thereafter, the amount of copper adsorbed showed a slight decrease (161.7 ± 1.4 mg g^{-1} EPS) at pH 7.5. On the other hand, the amount of lead adsorbed by the EPS increased from 145.8 ± 6.5 mg g^{-1} EPS at pH 4.5 to 181.2 ± 10.9 mg g^{-1} EPS at pH 5 and showed little variation till pH 6.5. A sharp increase in

the amount of lead adsorbed (218.8 ± 1.9 mg g^{-1} EPS) was observed at pH 7 followed by an immediate decrease at pH 7.5.

6.3.4 Effect of sodium chloride on metal-binding by EPS

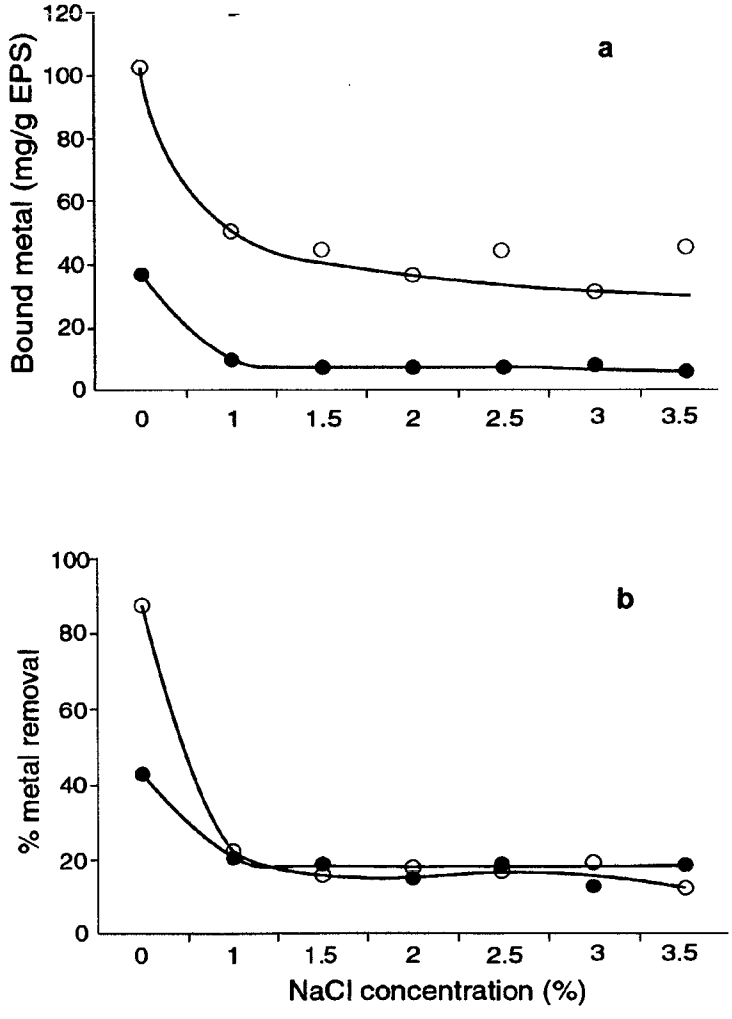

Fig. 6.5 Effect of sodium chloride concentration on the binding of metal ions by *Marinobacter* EPS expressed as (a) bound metal concentration (w/w) and (b) % metal ions removed.

The presence of sodium chloride drastically decreased the amount of copper and lead adsorbed. In the absence of sodium chloride, the amount of copper

and lead adsorbed was 256.9 ± 0.6 mg g^{-1} EPS and 86.8 ± 3.3 mg g^{-1} EPS, respectively. Thereafter, a sharp decrease in the copper adsorbed (125.3 ± 2.8 mg g^{-1} EPS) by the EPS was observed at 1% sodium chloride concentration (**Fig. 6.5a**). The copper concentration decreased with increasing concentration of sodium chloride to reach a minimal of 78.3 ± 6.6 mg g^{-1} EPS. The amount of lead adsorbed decreased from 86.8 ± 3.3 mg g^{-1} EPS to 22.3 ± 1.2 mg g^{-1} EPS with an increase in sodium chloride concentration.

The % removal of copper declined from 43% at 0% sodium chloride concentration to 21% at 1% sodium chloride and reduced to 13.1% at 3.5% sodium chloride concentration (**Fig. 6.5b**). On the other hand, % removal of lead decreased from 88% in the absence of sodium chloride to 22.5% in presence of 1% sodium chloride concentration and decreased to 12.8% at 3.5% sodium chloride concentration.

6.4 Discussion

Metal binding mechanisms have been explained as an interaction between the available binding sites and the free metal ions (Geesey and Jang 1990, Chen et al 1995) and follow the acid-base theory wherein the polymer acts as a Lewis base (electron donor) and the metal acts as a Lewis acid (electron acceptor) (Chen et al 1995). Although binding of metals is a complex process involving multiple binding sites, the one metal-one binding site model (one-metal sorption system) (Cabaniss et al 1984) has been used to explain the binding mechanism. In this system, it is assumed that there is no steric

interference during the binding process. Thus, the metal-polymer interaction is expressed as :

$M_e + L \overset{K_a}{\longleftrightarrow} M_eL$; (Loaëc et al 1997)

where M_e is free metal ion concentrations, L is the available ligand concentration, M_eL is metal-ligand complex and K_a is the affinity constant.

Microbial exopolysaccharides are known to bind metals to varying extents. Bacterial exopolymer used in the present study showed distinct differences in the ability to adsorb copper and lead (**Fig. 6.2a**). The amount of copper bound by the polymer (163 mg g^{-1} EPS) was 3 folds greater than lead (32.3 mg g^{-1} EPS). The maximum amount of copper bound by the *Marinobacter* EPS (189 mg g^{-1} EPS or 50.7 ± 1.7 mg g^{-1} carbohydrate) was greater than those bound by a crude exopolymers of freshwater sediment bacterium (31.2 mg g^{-1} carbohydrate) (Mittleman and Geesey 1985) and *Deleya marina* (16.8 mg g^{-1} EPS) (Ford et al 1987). In contrast to copper, lead was bound in small quantities. The maximum amount of lead bound by the bacterial polymer was only 41.4 mg g^{-1} EPS (**Fig. 6.2a**), which was much lower than those reported for EPS isolated from sludge (Lopez et al 1995) (64 mg g^{-1} EPS), fermentative yeast (601 mg g^{-1} EPS) (Volesky and May-Philips 1995) or deep-sea *Alteromonas* (316 mg g^{-1} EPS) (Loaëc et al 1997).

From the adsorption isotherms its clear that the bacterial EPS had greater affinity for copper than lead (**Fig. 6.3**). On the other hand, the maximum binding ability of bacterial EPS for copper was lower (181.8 nmol mg^{-1}

carbohydrate) than those reported by Mittleman and Geesey (489 nmoles mg^{-1} carbohydrate) (1985) and Ford et al (263 nmoles mg^{-1} carbohydrate) (1987). Similarly, the uptake capacity (Q_{max}) of *Alteromonas* EPS for lead was estimated to be 316 µg mg^{-1} carbohydrate (Loaëc et al 1997), which was much higher than those estimated for *Marinobacter* EPS (39.3 µg mg^{-1} EPS or 15.5 µg mg^{-1} carbohydrate) in this study. This implied that the amount of metal bound by *Marinobacter* EPS was much less than those reported for other bacterial EPS.

The % of metal removed declined with increasing metal ion concentration suggesting that the maximum binding took place at lower metal concentrations (**Fig. 6.2b).** Since metal-binding with a polymer is similar to acid-base reaction (Chen et al 1995), the availability of binding sites and the reactivity of the free metal ions play an important role in the effective removal of certain metals over others. For example, Loaëc et al (1997) found that *Alteromonas* EPS bound Pb more effectively than Cd and Zn. On the other hand, Ford et al (1987) found that copper ions were more effectively removed by crude bacterial EPS compared to Fe and Mn. One possible reason for such difference in their binding ability may be the ionic radii of the metal ions. The ionic radius of Pb (1.19 Å) is greater than that of copper (0.73 Å). For EPS having neutral charge, atoms having larger ionic radius are less preferred (Geesey and Jang 1990). It is therefore possible that the difference in the ionic radii might have resulted in the increased affinity of the polymer towards copper ions over lead. On the other hand, the poor binding of Pb might be attributed to the non-availability of proper binding sites. For example,

Pb prefers to bind with oxygen and nitrogen atoms that act as strong electron donor (Loaëc et al 1997). Both these donors are present in the form of hydroxyl groups (water), carboxylates (uronic acid), amides etc. The bacterial polymer used in the present study had higher protein content (44%) and relatively higher uronic acid content (17.6%), which can serve as good electron donors. However, the structural and spatial orientation of the binding groups (hyroxyl, carboxyl or amines) and their location (accessibility) might limit the binding of metals having larger ionic radius (Geesey and Jang 1990). Thus It is possible that larger ionic radii and poor accessibility to binding sites might be the reasons for the poor binding of Pb ions by the EPS.

The greater affinity towards copper might have ecological significance for the bacteria and might help in the immobilization of copper in the natural environment. Copper has been widely regarded as a toxic metal for microorganisms (Ford et al 1987). The production of an exopolymer with greater affinity to bind metals like copper may help in reducing the availability of copper in the free state. Bound metals are less available for bacterial uptake and thus help in the survival of bacteria in presence of copper (Ford et al 1986). On the other hand, the EPS has been shown to transport bound metal ions in to the sediments along with pore-water (Czajka et al 1997). Although the mobility of the bound copper or lead is not ascertained in this study, similar mechanisms might lead to accumulation and transport of heavy metals into the deeper sediment layers. This might increase the risk of contamination of ground water even when it is not directly exposed to these metals.

Binding of metals by organic compounds like bacterial EPS can be influenced by various factors including pH, polymer chemistry, other metal salts and solubility of the metals. Change in pH of the metal solution or the EPS solution can alter the efficiency of metal removal. In the present study, pH range from 4.5 to 7.5 was examined on the removal of copper and lead. Both copper and lead were more effectively bound with increasing pH (**Fig 6.4)** with maximal binding of copper and lead at neutral pH. Our findings were similar to the higher binding of metals reported at pH 7 than at pH 3 (Ferris et al 1989). Similarly, increased binding of both lead (Loaëc et al 1998) and copper (Lores and Pennock 1998) has been reported at near neutral pH. The chemistry of the polymer and the response of the binding sites to change in pH can influence the binding capacity of the bacterial polymer. Generally, binding of metals increases with increase in pH (Chen et al 1995). The active sites in the polymer are generally carboxyl groups, hydroxyl groups or amines that either get protonated or get ionized to become negatively charged with increase in pH. The *Marinobacter* EPS used in the present study was greater than 8 kDa MW in size and rich in proteins (44%) and uronic acid (17.6%). Since presence of carboxylic acids and amides are widely considered essential in any metal binding process (Loaëc et al 1998), a shift in pH can alter the overall binding mechanisms of the polymer. It has been shown that oxygen in hydroxyl and carboxyl groups of sugars like glucose, glucouronic acid can serve as weak electron donors during metal complexation process (Angyal 1989). Under low pH conditions, the active sites (oxygen) get protonated leading to a competition between protons and metals for the binding site. This

reduces the affinity for metals and thus leads to low binding of metals at pH 4 (Loaëc et al 1998). With increase in pH, there is reduced competition from free protons and the cations can now bind to a greater extent at the binding sites (Geddie and Sutherland 1990). On the other hand, binding sites having amides like $CONH^+$ can act as dual reactive sites. For example, under slightly acidic pH, $CONH^+$ become protonated and binding of metals like copper occurs mainly with oxygen, which serves as an electron donor (Kihn et al 1987). However, with the increase in pH, deprotonation occurs and nitrogen becomes electron donor and binds to the metal ions. Thus, a shift in pH might also alter the characteristics of the binding site thereby influencing the binding of metals.

Changes in the salinity or salt content of the solution are also responsible for changes in the binding of metals to polymer. The bacterial polymer showed maximal binding of lead and copper in the absence of sodium chloride and reduced drastically at 1% sodium chloride concentration (**Fig. 6.5a**). The reduction in binding of copper and lead in presence of 1% sodium chloride was by 74.3% and 51%, respectively (**Fig. 6.5b**). A similar decrease in chelation of metals like copper, cadmium, chromium and zinc by dissolved organic carbon with increasing salinity has been observed in estuarine waters (Lores and Pennock 1998). The increase in salt content in the metal solution may alter the binding due to complexation between the metals and salts. For example, salts of alkaline earth metals like Na, K, Ca and Mg are found in abundance in marine environment. In the dissolved state, the alkaline cations and their respective anions like chlorides are freely available and can interact

with other metals. Although copper and lead are known to displace alkaline earth metals bound to the polymer (Haug and Smidsrod 1970), these metal ions can also complex with anions of alkaline metal salts to form chloride and nitrate salts. Thus, fewer free ions are available to bind with the hydroxyl and carboxyl groups of the polymer (Lores and Pennock 1998). Anions like carboxylates in the polymer are known to possess the characteristics of selective preference for metals like copper over alkaline metals like Ca^{2+} (Haug and Smidsrod 1970). The availability of free carboxyl groups and its steric arrangement might therefore also be important for the preference of metals over alkaline metals. On the other hand, the small-scale variations in the bound metal concentration at higher sodium chloride concentrations showed that complexation of copper and lead with chloride ions alone was not responsible for the reduced metal binding at higher sodium chloride concentrations. If anion complexation was the only reason for low metal complexation with the polymers, increasing concentrations should lead to continuous decline in the binding of copper and lead. It appears that despite higher concentrations of free sodium ions, copper ions could still bind to the EPS. This could be possible only if there were binding sites in the EPS that were specific for copper or lead ions. This implies that although sodium ions could compete with copper or lead ions, it could not completely prevent the binding of copper and lead to the EPS.

6.5 Conclusions

Marinobacter EPS was found to selectively bind more copper than lead. The maximal % removal of either copper or lead took place at lower concentrations. The bacterial EPS was effective in removal of copper suggesting the probable ecological importance of the EPS against copper toxicity. The presence of sodium chloride showed marked reduction in the binding of copper and lead by the bacterial EPS. The variations in the binding of copper and lead under varying pH and salt concentrations indicate multiple binding sites for these metal ions. Since the bacterial EPS was assimilated by benthic polychaetes (Chapter 5), its ability to bind copper might lead to accumulation of copper in the tissues of the animals. Thus metals bound by the polymer in the natural environment might enter the food chain.

Summary

Summary

⇨ In this thesis, a study on the dynamics of three types of EPS viz. NaCl-soluble EPS, EDTA-soluble EPS and TEP was carried out at a shallow tropical station at the Dona Paula Bay, west coast of India during 1998-2000. During this study, factors like hydrography, nutrients, dry weight of particulate matter, Chl *a* (phytoplankton), POC and bacterial abundance were monitored to evaluate its influence on the seasonal variations of EPS.

⇨ The study site experienced three seasons- monsoon (June- September), post-monsoon (October to January) and pre-monsoon (February-May). Seasonal differences were observed in the concentrations of NaCl-soluble and EDTA-soluble forms of EPS in both suspended particulate matter and sinking macroaggregates. The EDTA-soluble EPS in the suspended matter was higher during monsoon. This coincided with an increase in suspended load, POC and nutrients but with a decreased phytoplankton (Chl *a*) biomass. On the other hand, NaCl-soluble EPS in the suspended matter was higher during post-monsoon months and coincided with higher Chl *a* concentrations. Unlike the two soluble forms of EPS, TEP concentrations at the study site remained unchanged during both monsoon and pre-monsoon months.

⇨ In the macroaggregate samples, NaCl-soluble EPS was higher during monsoon and coincided with low chl *a* concentration. On the other hand, EDTA-soluble EPS was higher during pre-monsoon months and coincided with increased POC concentrations. However, Chl *a* values in the macroaggregates did not change much seasonally.

⇨ The concentrations of all the three types of EPS in the suspended matter and macroaggregates had no correlation with changes in phytoplankton biomass suggesting that phytoplankton was not the major source of EPS at the sampling station. Moreover, factors like nitrate concentrations that regulate EPS production in phytoplankton cells did not correlate with EPS and TEP concentrations in the suspended matter.

⇨ The contribution of EPS (both NaCl and EDTA-soluble) to POC was < 5% in both water column and sinking macroaggregate samples. Similarly, TEP-C contribution to POC in the suspended matter was 6.9% but only 2% in the macroaggregates.

⇨ Since EPS and TEP are rich in organic carbon, their influence on the variation of ecto-glucosidase (both α– and β–) activity and bacterial production was also evaluated between 1999-2000. The total α-glc and β-glc activity was exceptionally high in the month of September 1999. On the other hand, higher total α-glc and β-glc activity was also recorded during the month of May 2000 in the macroaggregate samples. The higher

enzyme activity coincided with higher total bacterial production, Chl *a* and EDTA-soluble EPS.

⇨ Particle associated bacteria accounted for 49% and 61% of the total bacterial population in the suspended matter and macroaggregate samples, respectively. Similarly, a substantial fraction of total enzyme activity (85 to 95%) and bacterial production (75%) was associated with particles, which had significant linear correlation ($r= 0.663$, $p<0.01$) with EDTA-soluble EPS of the suspended matter. Such correlation highlights the significance of particle associated bacterial population in the turnover of EPS in the water column at the study site.

⇨ The total bacterial production and the glucosidase activity (both α- and β-forms) had positive linear trends with Chl *a* and EPS (both types) in both suspended matter and macroaggegates. Moreover, microbial activity of the macroaggregates showed significant correlations with Chl *a* and EPS of suspended matter. This indicates that the composition of suspended matter had a significant influence on the microbial activity in the macroaggregates.

⇨ TEP had no correlation with microbial activity in both suspended matter and macroaggregate samples, suggesting that TEP was not the preferred carbon source for bacterioplankton and probably served as a carbon sink at the study area.

⇨ The various aspects of microbial EPS were studied using a marine bacterium *Marinobacter* sp. Growth conditions for production of EPS by the bacterium were standardized. The EPS was isolated, partially purified and chemically characterized. *Marinobacter* EPS was richer in proteins compared to carbohydrates and also showed the presence of substantial amounts of residues like uronic acid and sulphates. Glucose was the dominant monosaccharide whereas aspartate was the major amino acid in the EPS.

⇨ EPS from *Marinobacter* sp. and a diatom *Skeletonema costatum* were used to evaluate its ability to produce large visible macroaggregates. Large macroaggregates (>15 mm^2 size) were produced at a faster rate in presence of bacterial EPS than diatom EPS. Significant differences were observed in the abundance (F_s= 28.07; $p<0.001$) and size (F_s= 9.15; $p<0.01$) of aggregates produced using EPS derived from two different sources. Interestingly, macroaggregate formation was not influenced by abundance of TEP or Coomassie-stained particles (CSP). Moreover, presence of killed bacteria decreased the formation of macroaggregates in both the experiments. Thus, it was clear that EPS was essential for the abiotic production of macroaggregates and could form particles even in the absence of TEP. Although macroaggregate production involves collision of particles, some organic particles- like killed bacterial cells in this study, might depress the aggregation process.

⇨ EPS from *Marinobacter* sp. was used to assess its suitability as a carbon source for heterotrophs. ^{14}C-glucose labeled *Marinobacter* EPS was coated on to sediments and fed to a benthic polychaete *Nereis* (*Hediste*) *diversicolor*. At the end of the feeding period, the animals ingested 27.85 dpm to 114.9 dpm of the labeled EPS. On an average, the absorption efficiency of the animal was 65.6%. However, substantial amount (51%) of the ingested EPS was respired as CO_2 and only 26% of the label was retained in the tissues after depuration. From this study, it was clear that the bacterial EPS served both as an energy and nutrient source to these benthic animals.

⇨ Natural bacterioplankton population collected from Dona Paula Bay was used to degrade the ^{14}C-glucose labeled *Marinobacter* EPS to evaluate its incorporation into the natural microbial population over a period of 11 days. During this experiment, the microbial cells were grown in aged seawater supplemented with labeled *Marinobacter* EPS as a source of organic carbon. A significant correlation ($r=0.993$, $p<0.001$) between the bacterial production and the incorporation of labeled EPS was observed during the course of the experiment. Both ^{14}C-incorporation and bacterial production were maximal in the first 24 h. A decline in ^{14}C-incorporation and bacterial production with time matched with the decline in monosaccharide (MCHO) concentrations and higher ecto-glucosidase activity. This suggests the utilization of MCHO by the natural marine bacterioplankton.

⇨ Metal binding properties of the bacterial EPS were evaluated using copper and lead as model substrates. The EPS bound both copper and lead to varying amounts. The polymer had a better binding capacity for copper than lead, which might be due to differences in the ionic radii of the metal ions, electro negativity of their complexes and accessibility of the binding sites. The binding was greater at near neutral pH for both copper and lead suggesting that the presence of carboxyl ions and/or $CONH^{+}$ groups might be responsible for the observed binding of copper and lead to the EPS. A substantial reduction in the amount of Cu or Pb ions bound by the EPS was observed in presence of sodium chloride, which might be due to competition for the binding sites between Na^{+} ions with Cu^{2+} and Pb^{3+}.

Although the significance of microbial EPS in the biogeochemical cycling of carbon and food-web is well-acknowledged, the chemical characteristics, properties, behavior and distribution of EPS may vary for several reasons. In this regard, the following could be concluded from the research work presented here:

i) EDTA-EPS supported the growth and enzyme activity of bacterial heterotrophs despite the relatively less contribution of the three different forms of EPS to the total organic carbon pool at Dona Paula Bay .

ii) *Marinobacter* EPS possessed both gelling and adsorptive properties, which was reflected in its ability to form large aggregates and bind metals.

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Appendix

APPENDIX-I A

a) Composition of Basal Salt Supplement (BSS) medium:

S. no.	Chemical	Concentration
1	Sodium chloride (NaCl)	30.0 g l^{-1}
2	Magnesium sulphate ($MgSO_4$. $5H_2O$)	7.0 g l^{-1}
3	Potassium chloride (KCl)	0.70 g l^{-1}
4	Ammonium chloride (NH_4Cl)	1.00 g l^{-1}
5	K_2HPO_4 (10%)	7.0 ml
6	KH_2PO_4 (10%)	3.0 ml
7	Trace metal solution*	100 ml
8	Distilled water	1000 ml
9	pH	7.6

b) Trace metal solution* composition:

S. no.	Chemical	Weight (g l^{-1})	Concentration (ppm)
1	H_3BO_3	2.85	0.5
2	$MnCl_2$. $4H_2O$	1.80	0.5
3	$FeSO_4$. $7H_2O$	2.49	0.5
4	Sodium tartarate	1.77	0.5
5	$CuCl_2$	0.0269	0.01
6	$ZnCl_2$	0.0208	0.01
7	$CoCl_2$	0.0404	0.01
8	$Na_2MoO_4.2H_2O$	0.0252	0.01

APPENDIX-IB

Composition of modified Basal Salt Supplement (BSS) medium

S. no.	Chemical	Concentration
1	Sodium chloride (NaCl)	30.0 g l^{-1}
2	Magnesium sulphate ($MgSO_4$. $5H_2O$)	10.0 g l^{-1}
3	Potassium chloride (KCl)	0.70 g l^{-1}
4	Ammonium chloride (NH_4Cl)	0.10 g l^{-1}
5	K_2HPO_4 (10%)	7.0 ml
6	KH_2PO_4 (10%)	3.0 ml
7	Trace metal solution	100 ml
8	Distilled water	1000 ml
9	pH	7.6

This modified BSS medium was supplemented with 1% glucose, low MW fractions of yeast extract (0.05%) and peptone (0.1%) while growing culture for EPS production. Concentrations of ammonium chloride, magnesium sulphated, phosphates and glucose were optimized.

APPENDIX-II

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

S. no.	Chemical	Concentration
	Stock Solution I	
1	Sodium nitrate ($NaNO_3$)	7.5 g
2	Sodium dihydrogen phosphate (NaH_2PO_4. H_2O)	0.5 g
3	Sodium silicate ($Na_2SiO_3.9H_2O$)	1.5 to 3 g
4	Distilled water	100 ml
	Stock solution IIa (100ml)	
5	Copper sulphate ($CuSO_4$. 5 H_2O)	0.98 g
6	Zinc sulphate ($ZnSO_4$.$7H_2O$)	2.20 g
7	Cobaltous chloride ($CoCl_2$.5 H_2O)	1.00 g
8	Manganous chloride ($MnCl_2$.4 H_2O)	18.0 g
9	Disodium molybdate ($Na_2MoO_4.2H_2O$)	0.63 g
	Stock solution IIb (1000 ml)	
10	Ferrous chloride ($FeCl.6H_2O$)	3.15 g
11	EDTA-sodium salt	4.36 g
	Stock solution III (vitamins)	
12	Thiamin.HCl	20.0 mg
13	Biotin (primary stock solution)	1.00 ml
14	Vitamin B_{12}	0.1 ml
15	Distilled water	100.0 ml

.....Continued

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 distilled water. The constituents of stock solution IIb were dissolved in 900 ml distilled 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. Alternatively, carbon dioxide was bubbled through the medium for 1 min before autoclaving. After autoclaving, the medium was immediately placed in crushed ice before using it for culturing the diatoms.
3) Stock solution of vitamins was filter sterilized before adding to the sterilized medium.