

## **CHAPTER 5**

## 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 DOC (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, Schlegel 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 ( $28 \pm 2^\circ\text{C}$ ) under static growth conditions.

#### Preparation of labelled EPS

After 72h of growth, 1ml of  $^{14}\text{C}$ -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  $-20^\circ\text{C}$ .

#### *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

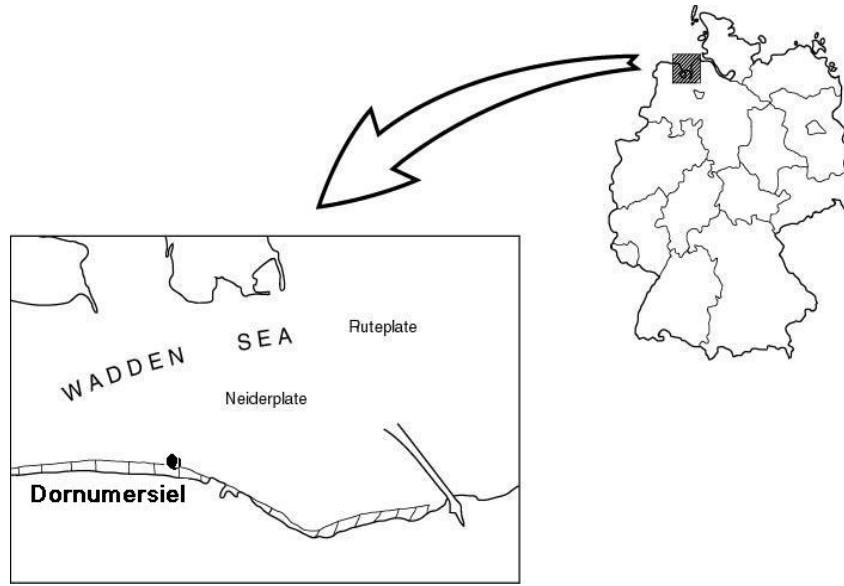
feeding upon detritus, mud, benthic microbial mats, macroalgae, sediment associated microflora and suspended phytoplankton (Riisgård 1991, Vedel and Riisgård 1993, Costa et al 2000).



**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  $\mu\text{m}$ ) 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. Sediments were sieved to obtain an average grain size in the range of 250-500  $\mu\text{m}$ . 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 ( $\text{H}_2\text{O}_2$ ) to oxidize the organic matter in the sediments. The sediment was then soaked in 0.5M NaOH solution containing 20 g  $\text{l}^{-1}$   $\text{Na}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{O}$ . 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  $\text{Na}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{O}$ . The rinsed

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

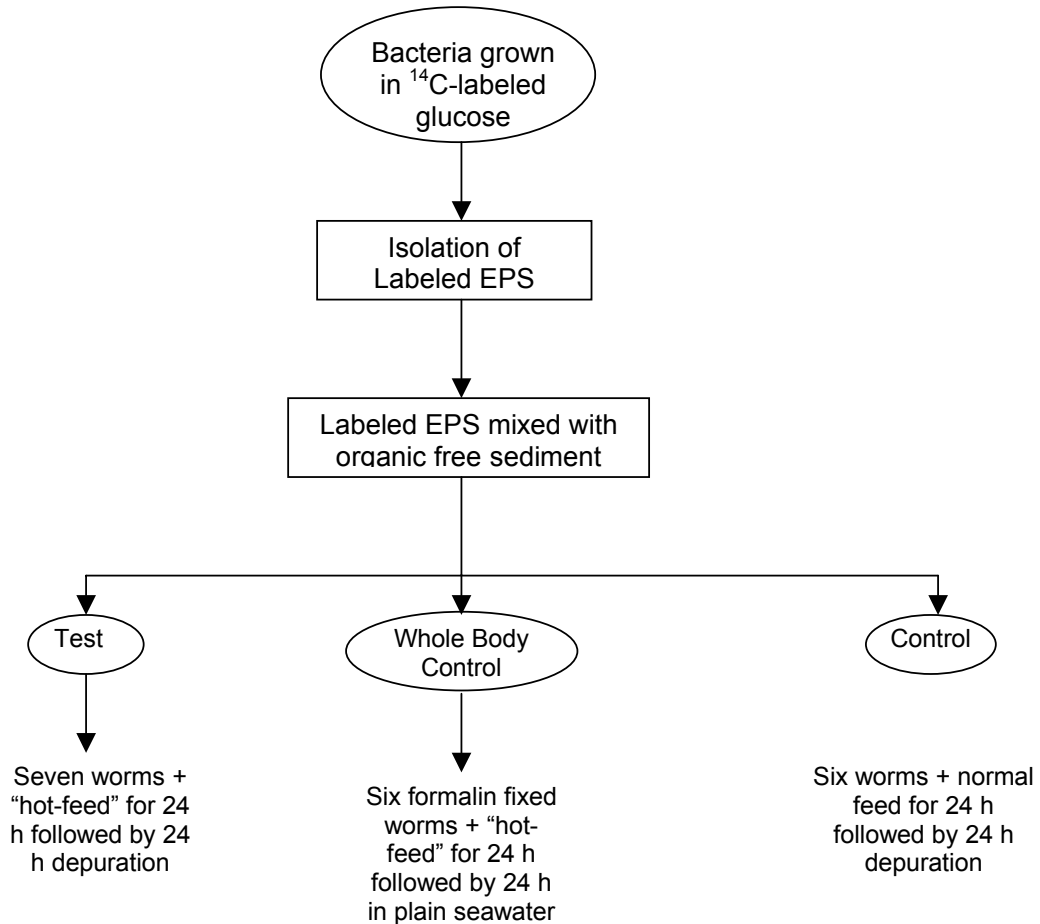
In order to prepare the feed, 50µl of the <sup>14</sup>C-labelled EPS solution (811.2 ± 258.2 dpm) was made to 100µl in sterile distilled water and mixed with ~300 mg of sediment as prepared above in micro centrifuge tubes. The sediment-polymer mix was incubated for 2 h at room temperature and then transferred 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 <sup>14</sup>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  $\mu\text{m}$ ) 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 (0.22 $\mu$ m) 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 (0.22 $\mu$ m). 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 to acidify the

phenylethylamine soaked papers. The flasks were kept overnight on a shaker to evolve carbon dioxide. The evolved CO<sub>2</sub> 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.

#### *<sup>14</sup>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

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

#### *5.2.1g % Assimilation efficiency*

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

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

and the total label ingested by the organism was estimated as

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

### *5.2.2 Uptake of bacterial EPS by natural bacterioplankton*

#### *<sup>14</sup>C-labeled bacterial EPS*

The <sup>14</sup>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<sup>-1</sup>.

#### *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 <sup>14</sup>C-labelled bacterial EPS (30480.7 dpm ml<sup>-1</sup>) was added, which gave an initial isotope concentration of 940 dpm flask<sup>-1</sup>. 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 as 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 ( $\alpha$ -glc and  $\beta$ -glc) was estimated on the sampling days except on day 3.

### Labelled EPS uptake and Bacterial production (Dual isotope measurements)

Both  $^{14}\text{C}$ -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  $\mu\text{l}$  of thymidine (10 nM thymidine strength) was added to each test tubes. The test-tubes were incubated for 120 min at  $28 \pm 2^\circ\text{C}$  in dark and shaken intermittently during the incubation period. At the end of the incubation period, the incorporation was terminated by adding 0.22  $\mu\text{m}$  filtered formalin (4% final concentration). The samples were then filtered through 0.22 $\mu\text{m}$  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  $^{14}\text{C}$ -EPS uptake

and thymidine incorporation on a Wallac scintillation counter. The amount of  $^{14}\text{C}$ -EPS incorporated was expressed as

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

wherein  $R_s$  and  $R_b$  are  $^{14}\text{C}$ -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 2B.

#### 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  $\mu\text{l}$  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<sub>ex.</sub> and 445 nm<sub>em</sub> (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  $\mu\text{m}$  filter paper to remove bacterial cells and were stored at  $-20^\circ\text{C}$  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 ( $\text{KBH}_4$ ) was added and incubated in dark for 4 h during which the monosaccharides were reduced to form alditols. After the incubation period, 0.05 ml 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 $^{14}\text{C}$ EPS to sediment

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

Individual worms weight (g)	Initial $^{14}\text{C}$ -label (dpm)	$^{14}\text{C}$ -ingested (dpm)	$^{14}\text{C}$ -residual (dpm)
297	266.4	95.5	170.9
628	162.8	72.8	90.0
656	231.9	27.9	204.0
845	234.8	77.1	157.7
850	297.4	91.5	205.9
1100	326.3	114.9	211.4
1165	174.0	64.4	109.6

**Table 5.1:** Ingestion of  $^{14}\text{C}$ -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  $^{14}\text{C}$ -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  $185.4 \pm 55.6$  dpm

During the feeding period, certain amounts of  $^{14}\text{C}$ -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  $^{14}\text{CO}_2$  (**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	<sup>14</sup> C-respired (dpm)	<sup>14</sup> C-excreted (dpm)	<sup>14</sup> 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	<sup>14</sup> C-respired (dpm)	<sup>14</sup> C-excreted (dpm)	<sup>14</sup> C-tissues (test) (dpm)	<sup>14</sup> 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  $48.6 \pm 29.5\%$  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  $26.2 \pm 10.8$  dpm or  $34.6 \pm 15.3\%$  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  $28.7 \pm 23.4\%$  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  $65.6 \pm 15.1\%$ . 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	% <sup>14</sup> C-respired	% <sup>14</sup> C-excreted	% <sup>14</sup> 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 <sup>14</sup>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 ( $F_s = 11.3$ ;  $p < 0.01$ ).

Parameters	Fecal matter	Residual	Tissue	Ingested
$^{14}\text{CO}_2$	-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  $^{14}\text{C}$ -labeled EPS and the total ingested  $^{14}\text{C}$ -labeled EPS.

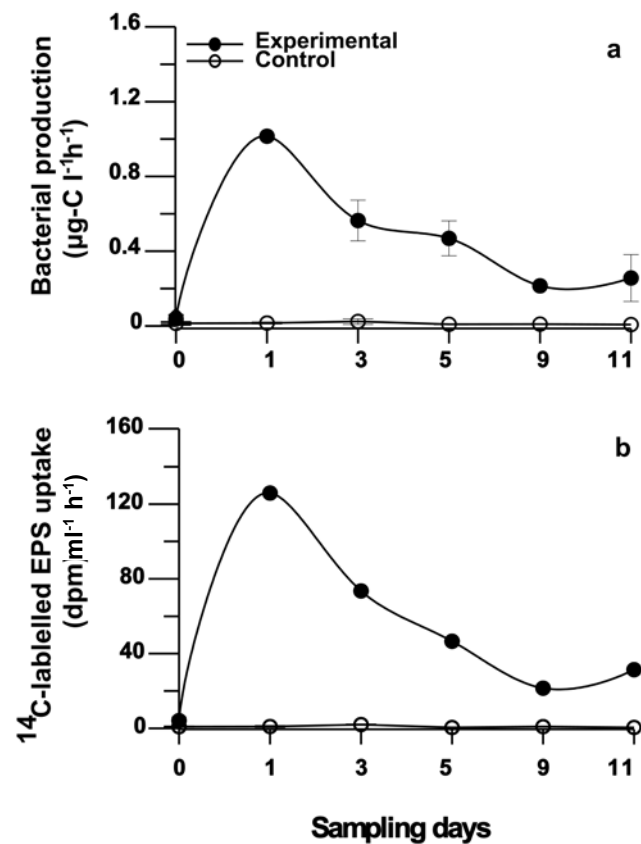
### 5.3.2 Heterotrophic uptake of bacterial EPS

#### Bacterial production and $^{14}\text{C}$ -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  $1 \pm 0.2 \mu\text{g-C l}^{-1} \text{h}^{-1}$  on day 1 to  $0.24 \pm 0.06 \mu\text{g-C l}^{-1} \text{h}^{-1}$  by day 11.

On the other hand, bacterial production in the controls did not change during the experiment and ranged from  $0.013 \mu\text{g-C l}^{-1}\text{h}^{-1}$  on day 0 to  $0.0069 \mu\text{g-C l}^{-1}\text{h}^{-1}$  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  $^{14}\text{C}$ -uptake rate was maximal on day 1 ( $126 \pm 0.8 \text{ dpm ml}^{-1} \text{ h}^{-1}$ ) and decreased to a low on day 9 ( $21.5 \text{ dpm ml}^{-1} \text{ h}^{-1}$ ) followed by a slight increase at the end ( $31.4 \pm 0.3 \text{ dpm ml}^{-1} \text{ h}^{-1}$ ). On the other hand, there were no observable differences in the uptake rates in controls.



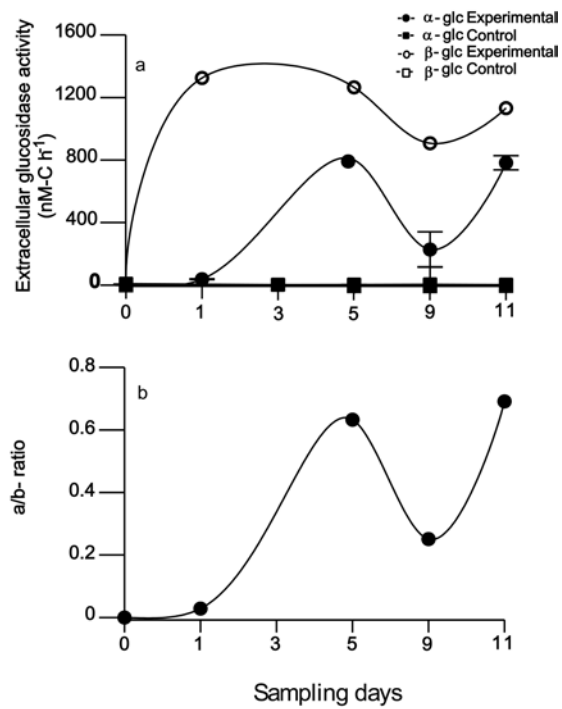
**Fig. 5.3** Temporal variations of a) bacterial production and abundance and b)  $^{14}\text{C}$ -EPS uptake in both experimental and control samples during the uptake of *Marinobacter* EPS by natural marine bacterioplankton. Each point is the mean of duplicate samples and error bar is the SD.

Extracellular glucosidase activity ( $\alpha$ - and  $\beta$ -glucosidase)

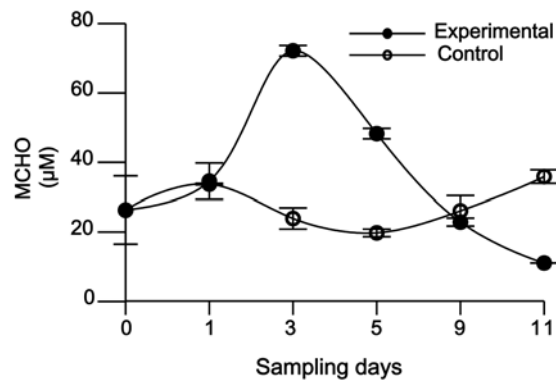
During the mineralization experiment,  $\beta$ -glucosidase enzyme activity was always higher than that of  $\alpha$ -glucosidase enzyme. However, the variation in enzyme activity was similar for both enzymes (**Fig. 5.4a**). On day 0, negligible activity was recorded in both  $\alpha$ -glc ( $5.6 \pm 0.4$  nM-C h<sup>-1</sup>) and  $\beta$ -glc ( $0$  nM-C h<sup>-1</sup>). However, a sharp increase in  $\beta$ -glc activity was registered ( $1324.8 \pm 106.5$  nM-C h<sup>-1</sup>) in the next 24 h. Thereafter, the  $\beta$ -glc activity declined marginally to  $907.3 \pm 0.8$  nM-C h<sup>-1</sup> on day 9 followed by an increase to  $1131.8 \pm 45.3$  nM-C h<sup>-1</sup> on day 11. In contrast,  $\alpha$ -glc activity did not increase much on day 1 ( $37.9 \pm 3.1$  nM-C h<sup>-1</sup>) followed by an increase to  $800.3 \pm 5.3$  nM-C h<sup>-1</sup> on day 5. Thereafter, the variations in  $\alpha$ -glc activity were similar to that of  $\beta$ -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  $26.3 \pm 9.8$   $\mu$ M on day 0 to  $72.2 \pm 1.5$   $\mu$ M on day 3 followed by a continuous decline until day 11 ( $11.1$   $\mu$ M) (**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 ( $26.3 \pm 9.8$   $\mu$ M) to day 5 ( $19.8 \pm 1.1$   $\mu$ M) followed by an increase until day 11 ( $35.9 \pm 1.9$   $\mu$ M).



**Fig. 5.4** Temporal variations in the activity of a)  $\alpha$ -glc activity and  $\beta$ -glc activity in both test and control samples and b)  $\alpha/\beta$ -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  $^{14}\text{C}$ -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  $\beta$ -glc activity. In contrast, the bacterial production and substrate uptake rates had weak relation with MCHO concentrations (**Table 5.7**).

Parameters	Bacterial production	MCHO	$\beta$ -glc activity	$\alpha$ -glc activity
$^{14}\text{C}$ -uptake	0.993***	0.418	0.666	-0.182
$\alpha$ -glc activity	-0.127	0.042	0.514	-----
$\beta$ -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  $\pm$  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 *Streblospio 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 ( $242 \pm 60.3$  dpm) on to the sediment was 25%, which was much higher than those recorded by Decho and Moriarty (1990). Unlike the bacterium-sized beads ( $< 2 \mu\text{m}$ ) 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 *Streblospio 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**). 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**). Its evident that the labeled EPS ingested by the animals was preferentially used for respiration and not incorporated into their tissues.

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 either due to ingestion or due to 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 ( $F_s = 11.3$ ,  $p < 0.01$ ) 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 *Streblospio 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 influences 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). Many metabolically active bacteria produce EPS (Heissenberger et al 1996). 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  $\beta$ -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  $^{14}\text{C}$ -incorporation and  $\beta$ -glc activity in the first 24 h suggests a close link between the  $^{14}\text{C}$ -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  $^{14}\text{C}$ -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  $^{14}\text{C}$ -labeled 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, it is 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  $\beta$ -glc activity on day 1 was greater than the  $\alpha$ -glc activity by an order of 10 and always remained greater than  $\alpha$ -glc activity. Moreover, the enzyme ratios ( $\alpha/\beta$ ) during the experiment were always less than 1. This indicates that  $\beta$ -glycosidic linkages were more abundant in the bacterial polymer. According to some

reports, higher  $\beta$ -glc activity is generally associated with less degradable organic matter (Müller-Niklas et al 1994). Chrost (1989) reported higher  $\beta$ -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  $\alpha$ -glc activity might be in response to the production of easily degradable lower molecular weight compounds released during the breakdown of EPS. On the other hand, the production of extracellular compounds by the growing heterotrophs might also induce the production of  $\alpha$ -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. Bacterial EPS were not ingested in large amounts and major portion of the ingested polymer was respired as carbon dioxide. *N. diversicolor* grown on a feed of labeled bacterial retained less than

30% of EPS in tissues. The ingestion and assimilation of EPS might 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**

## 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 1982a & b, Rudd et al 1984, Gaddie and Sutherland 1990, Loaec 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). 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 1982a & b, 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 an 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, Schlegel 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

### 6.2.1a Culture conditions of *Marinobacter* sp. and isolation of 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  $30^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  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  $\mu\text{m}$  filters, concentrated using a rotavapor at  $30^{\circ}\text{C}$  to less than 50 ml. This bacterial EPS solution was then precipitated overnight using ice-cold absolute ethanol at  $4^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$  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  $5.6 \pm 0.34$  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 ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and lead nitrate ( $\text{Pb}(\text{NO}_3)_2$ ) 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 Preparation of dialysis bags for metal-binding assay*

Prior to each assay, 8 kDa MW cut-off fresh dialysis bags (Spectrapor membrane tubings) were first washed with 10 mM EDTA in 1%  $\text{Na}_2\text{CO}_3$  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 ( $28 \pm 2^\circ\text{C}$ ). 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  $-20^{\circ}\text{C}$  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 &  $13\text{ mg l}^{-1}$ ) and lead (1.3, 1.9, 2.5, 4, 5, 6.4, 8, 10 &  $13\text{ mg l}^{-1}$ ). 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 ( $10\text{ mg l}^{-1}$ ) and copper ( $4\text{ mg l}^{-1}$ ) 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 ( $40 \text{ mg l}^{-1}$ ) and lead ( $100 \text{ mg l}^{-1}$ ) were diluted in wide-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  $4 \text{ mg l}^{-1}$  copper and  $10 \text{ mg l}^{-1}$  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 was replaced with distilled water, and treated in the same 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 ( $\text{mg}^{-1}$ ) against the available free ions using the equation

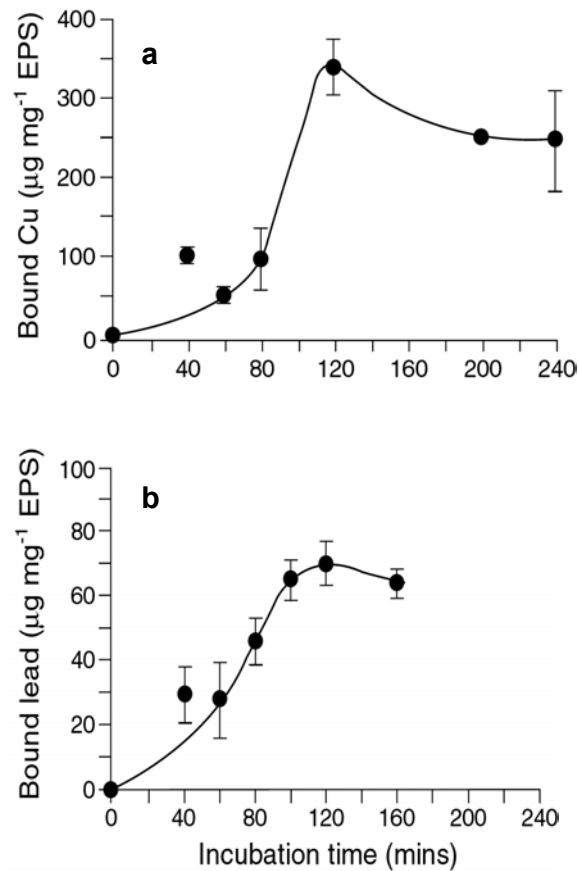
$$c/x = 1/k_1k_c + c/k_1; \text{ (Mittleman and Geesey 1985)}$$

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  $434.4 \pm 6 \text{ mg g}^{-1}$  EPS after 120 min following incubation. A similar adsorption pattern was observed for lead with the maximal adsorption ( $69.9 \pm 6.8 \text{ mg g}^{-1}$  EPS) following 120 min of incubation.

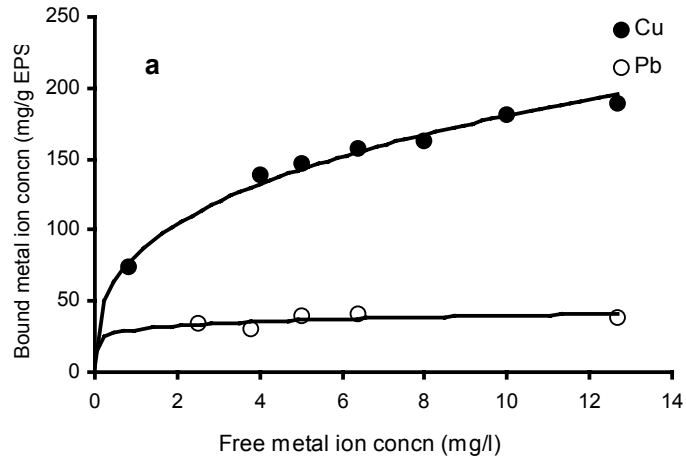


**Fig. 6.1** The changes in the amount of (a) copper and (b) lead bound by EPS produced by *Marinobacter* sp.

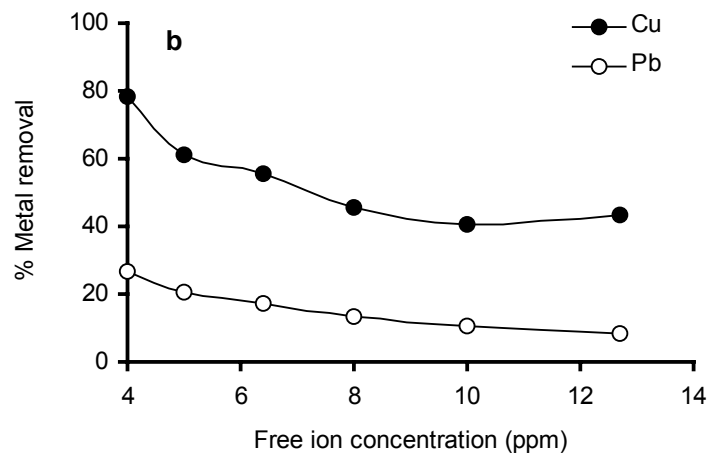
### 6.3.2 Effect of initial metal ion concentration on metal-binding by EPS

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 ( $39.3 \pm 1.9 \text{ mg g}^{-1} \text{ EPS}$ ) when the initial lead concentration was  $5 \text{ mg l}^{-1}$ . 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

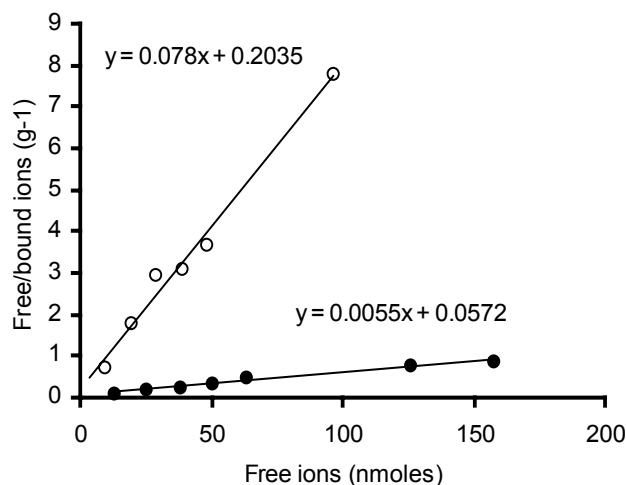


**Fig. 6.2a** Equilibrium sorption isotherms of lead and copper after binding by EPS of *Marinobacter* sp. The experiment was carried out at 220 rpm.



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

copper concentrations and varied from  $162.9 \pm 6.1 \text{ mg g}^{-1}$  EPS at  $4 \text{ mg l}^{-1}$  copper concentration to  $189 \text{ mg g}^{-1}$  EPS at  $10 \text{ mg l}^{-1}$  copper concentration. 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  $4 \text{ mg l}^{-1}$ , 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**).

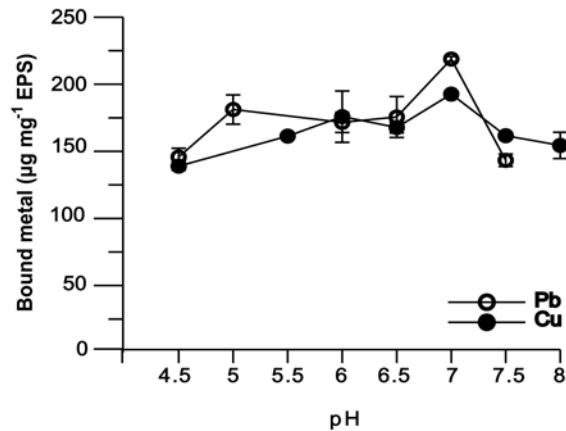


**Fig. 6.3** Langmuir's linear isotherms for copper and lead bound to crude exopolymer.

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  $\text{nmols mg}^{-1}$  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<sup>-1</sup> carbohydrate and 12.8 nmol mg<sup>-1</sup> carbohydrate, respectively.

### 6.3.3 Effect of pH on metal-binding by EPS



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

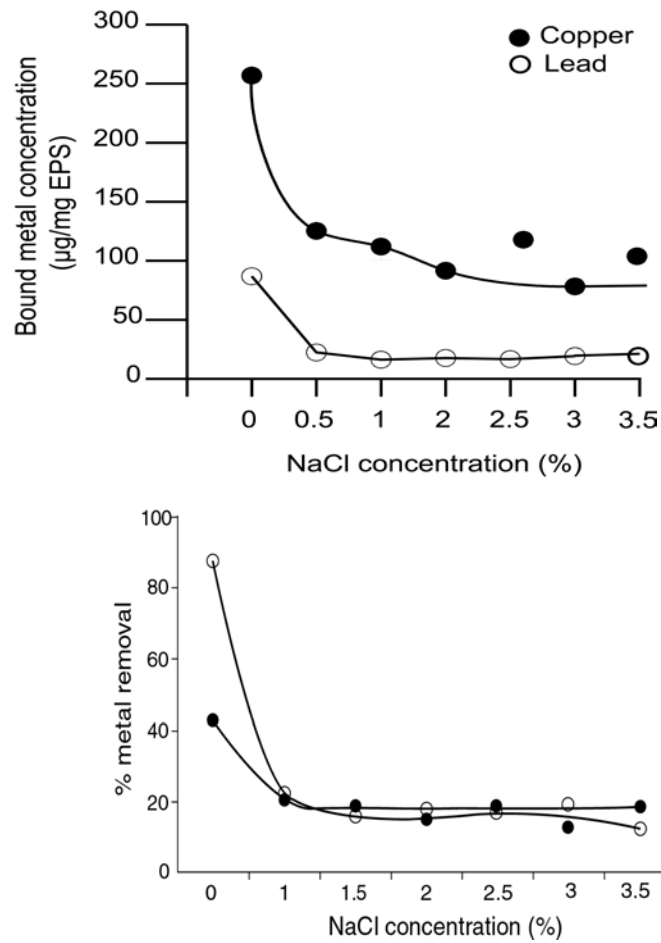
Both copper and lead were adsorbed more in near neutral pH than in acidic pH. The amount of copper adsorbed by the EPS showed an increase from 163 ± 6.1 mg g<sup>-1</sup> EPS at pH 4.5 to 192.7 mg g<sup>-1</sup> EPS at pH 7 (**Fig. 6.4**). Thereafter, the amount of copper adsorbed showed a slight decrease (161.7 ± 1.4 mg g<sup>-1</sup> 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<sup>-1</sup> EPS at pH 4.5 to 181.2 ± 10.9 mg g<sup>-1</sup> 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<sup>-1</sup> 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

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  $256.9 \pm 0.6 \text{ mg g}^{-1} \text{ EPS}$  and  $86.8 \pm 3.3 \text{ mg g}^{-1} \text{ EPS}$ , respectively. Thereafter, a sharp decrease in the copper adsorbed ( $125.3 \pm 2.8 \text{ mg g}^{-1} \text{ EPS}$ ) 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  $78.3 \pm 6.6 \text{ mg g}^{-1} \text{ EPS}$ . The amount of lead adsorbed decreased from  $86.8 \pm 3.3 \text{ mg g}^{-1} \text{ EPS}$  to  $22.3 \pm 1.2 \text{ mg g}^{-1} \text{ EPS}$  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.



**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.

## 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 :



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 ( $163 \text{ mg g}^{-1}$  EPS) was 3 folds greater than lead ( $32.3 \text{ mg g}^{-1}$  EPS). The maximum amount of copper bound by the *Marinobacter* EPS ( $189 \text{ mg g}^{-1}$  EPS or  $50.7 \pm 1.7 \text{ mg g}^{-1}$  carbohydrate) was greater than those bound by a crude exopolymers of freshwater sediment bacterium ( $31.2 \text{ mg g}^{-1}$  carbohydrate) (Mittleman and Geesey 1985) and *Deleya marina* ( $16.8 \text{ mg g}^{-1}$  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  $41.4 \text{ mg g}^{-1}$  EPS (**Fig. 6.2a**), which was much lower than those reported for EPS isolated from sludge (Lopez et al 1995) ( $64 \text{ mg g}^{-1}$  EPS), fermentative yeast ( $601 \text{ mg g}^{-1}$  EPS) (Volesky and May-Philips 1995) or deep-sea *Alteromonas* ( $316 \text{ mg g}^{-1}$  EPS) (Loaëc et al 1997).

From the adsorption isotherms it is 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 ( $181.8 \text{ nmol mg}^{-1}$  carbohydrate) than those reported by Mittleman and Geesey ( $489 \text{ nmoles mg}^{-1}$  carbohydrate) (1985) and Ford et al ( $263 \text{ nmoles mg}^{-1}$  carbohydrate) (1987). Similarly, the uptake capacity ( $Q_{max}$ ) of *Alteromonas* EPS for lead was estimated to be  $316 \text{ } \mu\text{g mg}^{-1}$  carbohydrate (Loaëc et al 1997), which was much higher than those estimated for *Marinobacter* EPS ( $39.3 \text{ } \mu\text{g mg}^{-1}$  EPS or  $15.5 \text{ } \mu\text{g mg}^{-1}$  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 ( $1.19 \text{ \AA}$ ) is greater than that of copper ( $0.73 \text{ \AA}$ ). 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 (hydroxyl, 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. 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  $\text{CONH}^+$  can act as dual reactive sites. For example, under slightly acidic pH,  $\text{CONH}^+$  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 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  $\text{Ca}^{2+}$  (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.