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Isolation and characterization of extracellular polysaccharides from the epipelic diatoms *Cylindrotheca closterium* and *Navicula salinarum*

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The production and composition of extracellular polymeric substances (EPS) in axenic batch cultures of the benthic marine epipelic diatoms *Navicula salinarum* and *Cylindrotheca closterium* were investigated. EPS was secreted into the medium and the bulk was loosely associated with the cells. Neither *N. salinarum* nor *C. closterium* formed a well-defined polysaccharide capsule. EPS of both *N. salinarum* and *C. closterium* consisted predominantly of polysaccharide but small quantities of protein were present as well. EPS also contained uronic acids and SO_4^{2-} groups. Analysis of monosaccharides using gas chromatography showed that for both species glucose and xylose were the main constituents, but several other monosaccharides were present in smaller quantities. Two fractions of EPS were distinguished: a small amount was secreted into the medium and a second fraction was extracted in water at 30 °C. For both species the two fractions differed somewhat in composition, indicating that they represented two different types of EPS. The EPS produced by *N. salinarum* and by *C. closterium* differed in their composition. The rate of EPS production in batch culture was highest during the transition from exponential growth to stationary growth. Negatively charged groups such as uronic acids and sulphated sugars determine the adhesion capacity of EPS and probably play an important role in the stabilization of intertidal sediments on which these diatoms grow and produce biofilms.

Key words: *Cylindrotheca closterium*, diatoms, EPS, extracellular polymeric substances, microphytobenthos, *Navicula salinarum*, polysaccharide, sediment stabilization

Introduction

Tidal mudflats are often colonized by dense communities of benthic diatoms. These organisms secrete copious amounts of extracellular polymeric substances (EPS) or mucilage (Decho, 1990; Hoagland *et al.*, 1993). Biofilms of diatoms and mucilage are formed on the sediment surface, and can increase the resistance of these sediments to erosion (Holland *et al.*, 1974; Dade *et al.*, 1990; Yallop *et al.*, 1994; Paterson, 1997). Mucilage production by benthic diatoms may therefore play an important role in the sediment dynamics of tidal mudflats.

It has been proposed that the secretion of EPS is involved in the mechanism of motility (Edgar & Pickett-Heaps, 1984). However, EPS may also perform several other functions such as protection against desiccation (Hoagland *et al.*, 1993). Nutrient and light availability are also likely to affect EPS production. Distinct effects of nutrients and light on exopolymer production have been reported for a wide range of phototrophic microorganisms such as *Chaetoceros affinis* (Myklestad *et al.*, 1989), *Chlamydomonas mexicana* (Kroen & Rayburn, 1984) and cyanobacteria (Bertocchi *et al.*, 1990).

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Although many studies have concerned the capsules of bacteria (Sutherland, 1977, 1988; Read & Costerton, 1987; Whitfield, 1988), little is known of the production and composition of EPS from epipelic diatoms (Hoagland *et al.*, 1993). Of special interest are anionic compounds such as carboxylic and SO_4^{2-} groups, which are determinant factors in the adhesion properties of EPS (Dade *et al.*, 1990). This study investigated the composition of EPS produced by two species of benthic diatoms, *Cylindrotheca closterium* and *Navicula salinarum*, grown in axenic batch cultures in the laboratory. A procedure for the extraction of EPS was developed leaving the cells intact. The cultures were characterized biochemically and growth and EPS production were monitored.

Materials and methods

Organisms and culture conditions

Strains of *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin and *Navicula salinarum* Grunow *in* Cleve & Grunow isolated from the Ems-Dollard estuary (The Netherlands) were kindly provided by H. Peletier (RIKZ, Haren, The Netherlands). Cells were grown in Kester medium (Kester

Table 1. Concentration of polysaccharides in culture supernatant from *Cylindrotheca closterium* and *Navicula salinarum*, following different parallel treatments of the cell pellet, and percentage recovery of cellular protein after extraction

Fraction	Extraction solvent	Extraction temp. (°C)	Polysaccharides (μg ml^{-1})	Cellular protein (% recovery)
C. closterium				
Supernatant	n.a.	n.a.	$4\cdot30 \pm 0\cdot26$	$99\cdot41 \pm 22\cdot73$
Pellet	Water	30	$3\cdot92 \pm 0\cdot34$	$105\cdot70 \pm 6\cdot31$
Pellet	0·5 M NaOH	20	$4\cdot73 \pm 0\cdot27$	$71\cdot39 \pm 9\cdot60$
Pellet	1 M NaCl	20	$0\cdot68 \pm 0\cdot30$	$98\cdot23 \pm 18\cdot99$
Pellet	0·1 M EDTA	20	$0\cdot58 \pm 0\cdot78$	$107\cdot90 \pm 23\cdot68$
N. salinarum				
Supernatant	n.a.	n.a.	$5\cdot06 \pm 1\cdot49$	$98\cdot19 \pm 6\cdot32$
Pellet	water	30	$4\cdot50 \pm 1\cdot20$	$94\cdot94 \pm 5\cdot88$
Pellet	0·5 M NaOH	20	$6\cdot88 \pm 1\cdot26$	$45\cdot49 \pm 4\cdot82$
Pellet	1 M NaCl	20	$0\cdot67 \pm 0\cdot61$	$106\cdot46 \pm 10\cdot22$
Pellet	0·1 M EDTA	20	$2\cdot26 \pm 0\cdot47$	$112\cdot10 \pm 11\cdot65$

Values are mean $\pm$ SD of three samples from a late log-phase culture. All pellet treatments were incubated for 1 h.
n.a., not applicable.

et al., 1967), with a salinity of 33 PSU. The diatoms were cultivated on a substratum of purified sea sand (Merck, Darmstadt, Germany) in 300 or 1000 ml glass Erlenmeyers at 20 °C. Cultures were illuminated at an incident irradiance of 60 μmol photons m^{-2} s^{-1}, produced by fluorescent tubes (Philips TLE 32/33) over a light:dark cycle of 12:12 h.

Cells were harvested either during exponential or during stationary growth, depending on the experiment. To harvest the cells the culture was gently shaken by hand, until all cells were in suspension. Subsequently, the sand was allowed to sediment before the cell suspension was poured off. Unless stated otherwise, samples were taken in triplicate from one culture. Axenity of the cultures was checked by plating on agar/medium and by microscopic observation.

Isolation of EPS

The cell suspension was centrifuged for 15 min at 20 000 *g* at 10 °C. Centrifugation at a lower temperature resulted in cell lysis. The pellet was extracted in water for 1 h at 30 °C. Tap water was used instead of distilled water to avoid cell lysis or leakage. This extract was subsequently centrifuged for 15 min at 20 000 *g* and 10 °C. Polymers were isolated from the culture medium as well as from the warm water extract by overnight precipitation in cold (−20 °C) 80% (v/v) ethanol. Because EPS was present in small amounts in the culture supernatant, this fraction had to be pre-concentrated by ultrafiltration using a 3 kDa cut-off membrane (Filtron) prior to precipitation in ethanol for compositional analyses. The precipitated EPS was dried under a flow of nitrogen gas and subsequently stored dry at −20 °C before analysis. The fraction obtained after centrifugation of a cell suspension was designated 'non-attached EPS', while the fraction obtained by extraction in water for 1 h at 30 °C was designated 'attached EPS'.

Prior to establishment of these extraction steps as the optimal procedure, several other treatments of the pellet remaining after centrifugation had been tested. All extraction procedures were followed by microscopy to check for cell lysis. Damaged or lysed cells could be recognized as empty frustules or as cells from which the protoplasm had leaked out. In addition, the cellular content of protein was measured before and after extraction to establish whether any losses occurred during the extraction. All extracts were obtained by centrifugation for 15 min at 20 000 *g*. From all extracts polysaccharides were isolated by overnight precipitation in cold (−20 °C) 80% (v/v) ethanol and quantified with the phenol/H$_2$SO$_4$ assay (Herbert *et al.*, 1971). In one series of experiments, cell pellets from late log-phase cultures were extracted in four consecutive steps using commercially available artificial seawater (Instant Ocean). Extraction lasted for 1 h at 20 °C. In this experiment, polysaccharides were found in the culture supernatant (medium) at a concentration of 4·7 and 7·1 mg ml^{-1} for *C. closterium* and *N. salinarum*, respectively. The four additional centrifugation steps in Instant Ocean resulted in very low additional amounts of polysaccharides. However, Alcian Blue staining clearly revealed that EPS was still associated with the cells.

In another series of experiments, pellets were extracted by the following solutions: (i) water, (ii) 0·05 M NaOH, (iii) 1 M NaCl or (iv) 0.1 M EDTA, either for 1 h at 30 °C (i) or for 1 h at 20 °C (ii–iv). Extraction with 0·1 M EDTA or 1 M NaCl yielded much less polysaccharide than extraction in water (Table 1). Extraction with 0·5 M NaOH resulted in cell lysis. To distinguish between the effects of temperature and water, pellets were subjected to extraction for 1 h in water at 20 °C or 30 °C or to extraction in the growth medium at 30 °C (Table 2). Incubation of the cell pellet with medium at 30 °C yielded much less polysaccharide compared with incubation in water (Table 2). In water, the elevated temperature of 30 °C increased the amount of polysaccharides. Extraction in water at 70 °C resulted in cell lysis (results not shown).

Table 2. Concentration of polysaccharides in culture supernatant from *Cylindrotheca closterium* and *Navicula salinarum* and following different parallel treatments of the cell pellet

Fraction	Extraction solvent	Extraction time (min)	Extraction temperature (°C)	Polysaccharide (μg ml^{-1})	
				C. closterium	*N. salinarum*
Supernatant	n.a.	n.a.	n.a.	$5 \cdot 02 \pm 1 \cdot 67$	$2 \cdot 70 \pm 0 \cdot 90$
Pellet	Medium	60	30	$0 \cdot 00 \pm 0 \cdot 02$	$0 \cdot 25 \pm 0 \cdot 62$
Pellet	Water	0	n.a.	$3 \cdot 23 \pm 0 \cdot 68$	$5 \cdot 81 \pm 1 \cdot 93$
Pellet	Water	60	20	$4 \cdot 58 \pm 0 \cdot 40$	$7 \cdot 94 \pm 1 \cdot 11$
Pellet	Water	60	30	$6 \cdot 10 \pm 0 \cdot 69$	$9 \cdot 68 \pm 1 \cdot 60$

Values are mean $\pm$ SD of three samples from a late log-phase culture.

n.a., not applicable.

Hence, incubation of the pellet in water for 1 h at 30 °C resulted in the maximum amount of EPS, leaving the cells intact (Table 2). Microscopic observation of Alcian Blue-stained pellets after water extraction showed that all EPS had been removed.

Cell analyses

Cells were counted using a Coulter counter as well as by microscopy using a Bürker-Türk counting chamber (counting a minimum of 300 cells). Approximate biovolume values were obtained by measuring cells and microscopically applying the formulae from the BIOVOL program developed by D. B. Kirschtel.

Cell dry weight was determined in duplicate by filtration of 20 ml of cell suspension on pre-weighed Whatman GF/C filters. The filters were dried overnight at 80 °C to constant weight.

Cell protein was determined on freeze-dried cell pellets by the Lowry method (Herbert *et al.*, 1971) using bovine serum albumin (BSA) as reference.

Pigments were extracted from freeze-dried cell pellets by 90% (v/v) acetone (20 °C, 1 h in the dark), and subsequently separated and quantified using HPLC as described in Van der Staay *et al.* (1992).

Intracellular carbohydrate was determined on freeze-dried cells from which all EPS had been removed, applying the extraction procedure as described. Generally, the phenol/H$_2$SO$_4$ assay was preferred as a 'total carbohydrate' assay (Herbert *et al.*, 1971). However, the phenol/H$_2$SO$_4$ assay appeared less suitable for analysis of whole algal cells. Therefore, total hexose content was determined with Anthrone reagent using glucose as standard (Herbert *et al.*, 1971). Storage polymers were analysed by alkaline hydrolysis of the cells by 30% (w/v) KOH at 100 °C for 90 min, followed by overnight precipitation in cold (-20 °C) 80% (v/v/) ethanol (Ernst *et al.*, 1984). Precipitated and non-precipitated intracellular carbohydrate was dried under a flow of nitrogen gas and enzymatically hydrolysed using a mixture of 24 units α-amylase (Sigma) and 12 units amyloglucosidase (Boehringer) per millilitre of acetate buffer (100 mM, pH 4·75) at 20 °C for 1 h. Glycogen was used as a reference from which the recovery was calculated. Another batch of precipitated and non-precipitated carbohydrate was treated with 0.005 units β-glucanase (Fluka) per ml acetate buffer (50 mM, pH 5) at 38 °C for 16 h using laminarin (β-1,3 glucan) as reference. Free glucose and glucose liberated after enzymatic hydrolysis were measured with the GOD assay (Boehringer).

EPS analyses

The total carbohydrate content of extracted EPS was estimated using the phenol/H$_2$SO$_4$ assay with glucose as standard (Herbert *et al.*, 1971). Uronic acids were assayed using the method of Blumenkrantz & Asboe-Hansen (1973) with glucuronic acid as standard. Sulphated sugars were determined by measuring SO$_4$$^{2-}$ according to the method of Terho & Hartiala (1971) after hydrolysis of the polymer. MgSO$_4$ was used as standard. The protein content of the EPS was determined according to Bradford (1976). BSA was taken as standard.

The monosaccharide composition of EPS was identified by gas chromatographic analysis. EPS and intracellular carbohydrates were hydrolysed and methylated with acidic methanol containing 2 M HCl. Hydrolysis was performed for 16 h at 85 °C. The resulting methyl-glycosides were converted to their trimethylsilyl derivatives and separated on a WCOT fused silica column (25 × 0·32 mm) in a Chrompack CP9000 gas chromatograph equipped with a split-splitless injector and a flame ionization detector (FID) (Chaplin & Kennedy, 1986). The temperatures of injector and detector were 220 and 250 °C, respectively. The carrier gas was oxygen-free dry helium at a flow rate of 25–30 ml min^{-1}. The make-up flow was 25–30 ml min^{-1}. Mannitol was used as internal standard.

For microscopic observation of anionic polysaccharides cell suspensions were dried on glass microscope slides and stained using Alcian Blue (3% in acetic acid, pH 2·5) for 30 min, then rinsed in water and dried. Sulphated sugars were stained using Alcian Blue (3% in 0·5 M HCl, pH 0·5) (Crayton, 1982).

Results

Growth was measured as the increase in biomass (protein) as well as cell number (Figs 1, 2). Protein concentration reached stationary phase after approximately 5 days, whereas cell number continued to increase. This was reflected in the decreasing protein content of the cells (Figs 1, 2). The generation times during exponential growth of *C. closterium* and *N. salinarum* were 14·9 and 29·9 h,
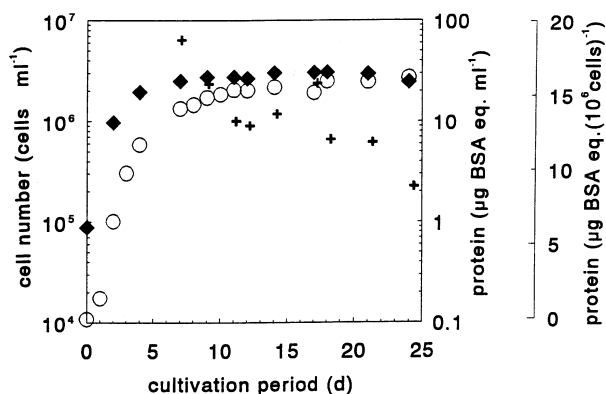

Fig. 1. Typical growth curve of *Cylindrotheca closterium*. Circles, cell number; diamonds, protein; crosses, protein content of the cells.
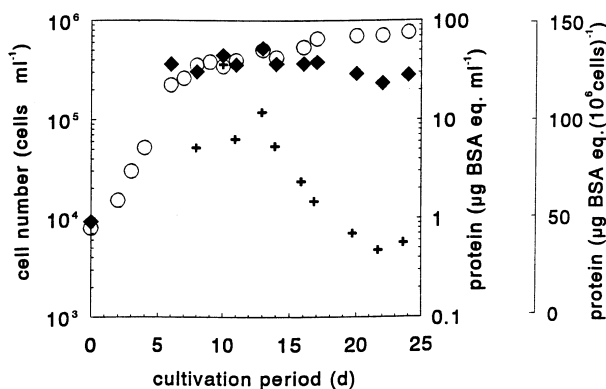

Fig. 2. Typical growth curve of *Navicula salinarum*. Circles, cell number; diamonds, protein; crosses, protein content of the cells.

Table 3. Cellular composition of late log-phase cultures of *Cylindrotheca closterium* and *Navicula salinarum*

	Concentration (μg 10^6 cells)	
	C. closterium	*N. salinarum*
Dry weight	840 ± 50	1700 ± 110
Protein[a]	22·80 ± 1·10	67·00 ± 4·70
Carbohydrate[b]	4·88 ± 0·29	17·11 ± 2·38
Chlorophyll *a*[c]	0·41 ± 0·11	4·53 ± 1·81
Chlorophyll *c*[c]	0·01 ± 0·00	0·15 ± 0·08
Fucoxanthin[c]	0·29 ± 0·08	2·11 ± 0·16
Diadinoxanthin[c]	0·05 ± 0·02	0·20 ± 0·08
β-carotene[c]	0·01 ± 0·00	0·03 ± 0·02

Values are mean ± SD of three samples from a late log-phase culture.
[a] Analysed using the Folin reagent (Herbert *et al.*, 1971).
[b] Analysed using the Anthrone reagent (Herbert *et al.*, 1971).
[c] Analysed with HPLC after extraction with acetone (Van der Staay *et al.*, 1992).

equalling growth rates of 0·067 and 0·033 h^{-1}, respectively. The maximum growth rates, μ_{max}, of both cultures based on the increase in protein were 0·047 and 0·045 h^{-1} for *C. closterium* and *N. salinarum*, respectively. The lag phase was 1–2 days and exponential growth lasted only about 4 days for both species. The cultures were harvested at the end of the log phase of growth and the cellular composition was determined (Table 3). The cell composition (μg component 10^{-6} cells) showed much higher quantities of constituents per cell in *N. salinarum* compared with *C. closterium*. This was partly attributed to the fact that the volume of cells of *N. salinarum* was about twice as great as of those of *C. closterium* (approximately 200 and 100 μm^3, respectively), which was reflected in the dry weight of the cells. The weight of the cells was relatively high due to the silica frustule enveloping the cell. Even when taking its larger cell volume into account, *N. salinarum* contained higher amounts of protein, twice as much carbohydrate and about 5 times as much photosynthetic pigments compared with *C. closterium*.

The storage carbohydrate of both species was identified by enzymatic hydrolysis as a β-1,3-linked glucose polymer. This polymer was water-soluble and did not precipitate in cold 80 % ethanol.

Microscopic observation of cells stained with Alcian Blue showed that carboxylated and sulphated polysaccharides were present. It was observed that EPS was associated with the cells but did not form a well-defined capsule. In addition, some material was diffusely secreted into the medium and was not associated with the cell.

EPS isolated from cultures of *C. closterium* and *N. salinarum* that had entered the stationary phase of growth was characterized. In both species, attached and non-attached EPS were predominantly composed of neutral carbohydrates (Table 4). EPS produced by *C. closterium* contained a small amount of protein. In *N. salinarum* only non-attached EPS contained a small amount of protein. The amount of uronic acids was about 8 % in *N. salinarum* and was the same in the attached and non-attached fraction. In *C. closterium* attached EPS contained 21 % uronic acid, while non-attached EPS contained only 5 %. In both species attached EPS contained about 11 % sulphate. Whereas in non-attached EPS of *C. closterium* sulphated sugars were absent, this fraction of *N. salinarum* contained 6 % of these sugars.

Table 5 lists the monosaccharide composition of both fractions of EPS of *N. salinarum* and *C. closterium* as well as intracellular carbohydrate. Attached EPS was composed predominantly of glucose with minor quantities of other sugars. There were minor differences in the sugar composition of attached EPS of both species. The composition of non-attached EPS was different from attached EPS, containing much less glucose. In *N. salinarum*, glucose content of non-attached EPS was 42 % and in *C. closterium* it was only 23 %. Non-attached EPS from the latter species contained a very high proportion of xylose (46 %), which was also a dominant sugar in non-attached EPS from *N. salinarum* (20 %). Galactose was also

Table 4. Composition (weight %) of attached and non-attached EPS isolated from late log-phase cultures of *Cylindrotheca closterium* and *Navicula salinarum*

	C. closterium		N. salinarum	
Component	Non-attached	Attached	Non-attached	Attached
Carbohydrates[a]	$86 \cdot 0 \pm 22 \cdot 0$	$60 \cdot 4 \pm 6 \cdot 2$	$80 \cdot 8 \pm 12 \cdot 2$	$80 \cdot 3 \pm 36 \cdot 6$
Protein	$9 \cdot 2 \pm 5 \cdot 1$	$7 \cdot 7 \pm 3 \cdot 3$	$4 \cdot 9 \pm 0 \cdot 9$	$0 \cdot 5 \pm 0 \cdot 2$
Uronic acids	$4 \cdot 8 \pm 0 \cdot 4$	$21 \cdot 0 \pm 1 \cdot 7$	$8 \cdot 0 \pm 1 \cdot 8$	$7 \cdot 7 \pm 1 \cdot 3$
SO_4^{2-}	0	$10 \cdot 9 \pm 0 \cdot 3$	$6 \cdot 3 \pm 1 \cdot 8$	$11 \cdot 5 \pm 2 \cdot 2$

Values are mean $\pm$ SD of three samples from a late log-phase culture.
[a] Analysed using the phenol/H_2SO_4 assay.

Table 5. Monosaccharide composition (mole %) of attached and non-attached EPS and of intracellular carbohydrates from late log-phase cultures of *Cylindrotheca closterium* and *Navicula salinarum*

	C. closterium			N. salinarum		
Monosaccharide	Non-attached ($n = 4$)	Attached ($n = 5$)	Cellular[a] ($n = 2$)	Non-attached ($n = 4$)	Attached ($n = 6$)	Cellular[a] ($n = 2$)
Glucose	22·9	82·5	27·5	41·6	85·1	79·1
Galactose	12·2	2·2	16·8	19·1	4·3	0
Mannose	4·1	7·6	25·4	13·8	0·5	8·3
Rhamnose	14·7	1·0	14·0	5·6	9·1	7·3
Arabinose	0	2·8	0	0	0·6	0
Xylose	46·1	3·9	16·3	20·2	0·3	5·4

Mean of n replicate samples from a late log-phase culture.
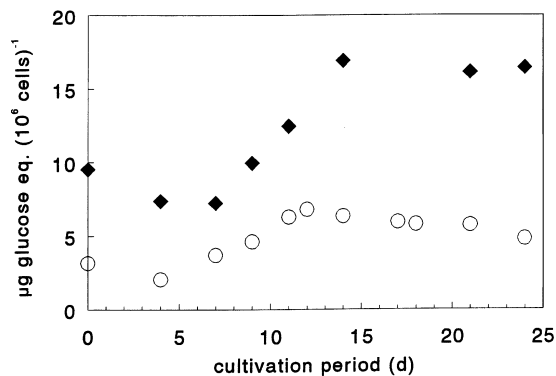[a] Total carbohydrates of cells after EPS extraction.

Fig. 3. Changes in concentrations of non-attached EPS (circles) and attached EPS (diamonds) during batch growth of *Cylindrotheca closterium*, expressed on a per cell basis.
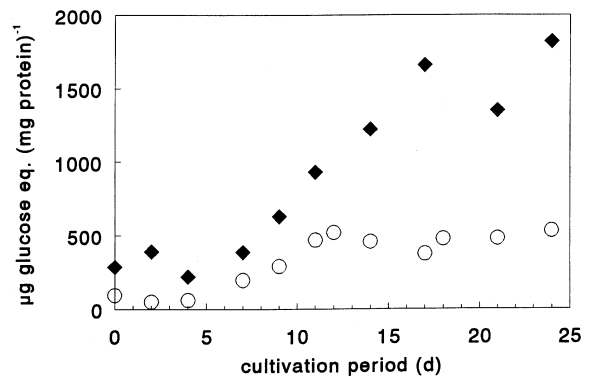

Fig. 4. Changes in concentrations of non-attached EPS (circles) and attached EPS (diamonds) during batch growth of *Cylindrotheca closterium*, expressed per unit protein.

an important component sugar in non-attached EPS. Other important sugars in non-attached EPS in both species were mannose and rhamnose. Arabinose was found in both species exclusively in attached EPS. Composition of intracellular carbohydrate showed a number of differences between the two species. In *C. closterium* intracellular carbohydrate contained only 27% glucose, whereas galactose, mannose, rhamnose and xylose were present in relatively large quantities. In contrast, in *N. salinarum* 79% of intracellular carbohydrate was composed of glucose, while galactose could not be detected and the other sugars were present in minor amounts.

In *C. closterium* the concentration of attached EPS, expressed on a per cell basis, increased when the culture entered the stationary phase of growth, from day 5 onwards, and remained constant after day 14 (Fig. 3). The amount of non-attached EPS per cell, however, increased only slightly. The ratio of attached EPS to protein increased much more than attached EPS per cell (Fig. 4). This was due to the fact that the amount of protein per cell dropped from day 8 onwards (Fig. 1). Regular microscopic observations revealed that cells became smaller by approximately 20% during cultivation. In *N. salinarum*, EPS concentrations were below the limit of detection until
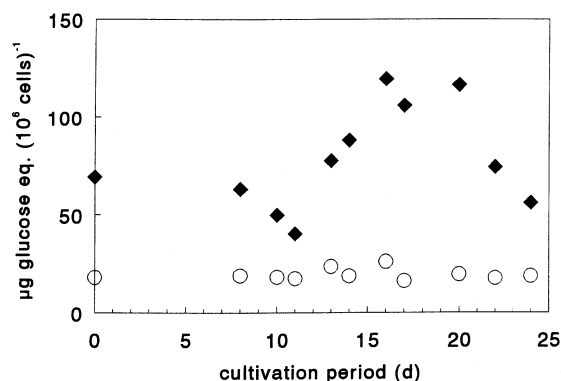
Fig. 5. Changes in concentrations of non-attached EPS (circles) and attached EPS (diamonds) during batch growth of *Navicula salinarum*, expressed on a per cell basis.
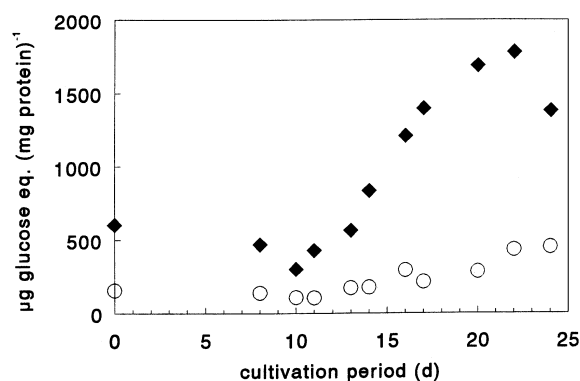

Fig. 6. Changes in concentrations of non-attached EPS (circles) and attached EPS (diamonds) during batch growth of *Navicula salinarum*, expressed per unit protein.

day 8 (Figs 5, 6). Analysis could only be performed on the pre-culture that was used for the inoculation of the experimental culture (day 0). The pattern of EPS secretion in *N. salinarum* was very similar to that in *C. closterium*. Attached EPS began to accumulate when the culture entered the stationary phase, remained constant after day 16 and eventually decreased (Fig. 5). This decrease in attached EPS was not observed in *C. closterium*. In *N. salinarum* non-attached EPS per cell remained constant throughout growth (Fig. 5). However, when expressed on a protein basis, a slight increase was observed (Fig. 6). This was due to the decrease in the cellular protein content. In contrast to *C. closterium*, cell volume in *N. salinarum* did not decrease. The decreasing protein content was also responsible for the fact that attached EPS expressed on a protein basis continued to increase longer and showed a less dramatic decrease at the end of the experiment (Fig. 6).

Discussion

Growth of the two benthic epipelic diatoms *Cylindrotheca closterium* and *Navicula salinarum* in batch culture showed a typical pattern. Growth was rapid and both cultures entered the stationary phase 4–5 days after inoculation. Cell division was not synchronized with protein synthesis in *N. salinarum*. It lagged behind during exponential growth and caught up during the stationary phase. In *C.*

closterium cell numbers still increased slightly when protein was constant. This may partly be attributed to a reduction in cell size, although this reduction could only partly account for the observed decrease protein content. These results emphasize the fact that relating measured cell parameters either to cell number or to biomass (protein) may lead to different conclusions, depending on the growth phase during which the culture was harvested. The yield of biomass in both cultures at the end of the experiment was the same (approx. 20–25 μg protein ml^{-1}), suggesting that nutrient depletion cause cessation of growth. The decreasing protein content of the cells indicated that cells may have become nitrogen-starved.

When comparing the two species, cell size should be taken into account. The cell volume of *N. salinarum* was about twice that of *C. closterium* at the transition of the log phase of growth to the stationary phase. The dry weight of the two diatoms reflected their (2-fold) difference in size. The much higher content of all photopigments in *N. salinarum* is the consequence of its greater size, more pigments being needed to achieve a similar biomass-related light absorption as in *C. closterium*. However, *N. salinarum* contained proportionally more protein and intracellular carbohydrate than *C. closterium*. This probably allowed *N. salinarum* to continue cell division after growth had ceased, without decreasing cell size. This could provide *N. salinarum* with an important ecological advantage over *C. closterium*, but this aspect was not the subject of this study. The objective of this study was rather to investigate the consequences of these differences in growth, cell size and biochemical composition for EPS production.

EPS produced by marine benthic motile diatoms does not form stalks or capsules like those of many freshwater benthic diatoms, but a highly hydrated matrix of strands in which cells and sediment particles are embedded (Hoagland *et al.*, 1993). This property of EPS of epipelic marine diatoms allows it to be extracted from the cells by a relatively mild method. By comparison, capsular polysaccharides of the marine fouling diatom *Navicula subinflata* were extracted with 1 M NaCl by Bhosle *et al.* (1995) and capsular EPS of the freshwater diatom *Navicula pelliculosa* was water-insoluble and could only be extracted by using 20% NaOH (Lewin, 1955). Capsular material from the cyanobacterium *Microcystis aeruginosa* was isolated using repeated ultrasonication and centrifugation (Nakagawa *et al.*, 1987). Bertocchi *et al.* (1990) proposed hot water extraction as a general method for cyanobacterial EPS. A wide variety of extraction methods have been applied for bacterial EPS. These include extraction by NaOH, EDTA and H_2SO_4, as well as high-speed centrifugation and ultrasonication (Brown & Lester, 1980; Novak & Haugan, 1981; Rudd *et al.*, 1982). Since *C. closterium* and *N. salinarum* did not possess a well-defined capsule, the EPS which was associated with the cells as a loose matrix could be quantitatively extracted by water and subsequent centrifugation. Any other method that was tested either resulted in a significantly lower yield or

damaged the cells. Extraction with water did not damage the cells. This was supported by microscopic observations, by the low concentrations of protein found in the extracted EPS, and by the fact that there was no loss of protein from the pellets. In addition, leakage of carbohydrates from the cells due to osmotic stress did not seem to have occurred, since virtually all the carbohydrates extracted were polysaccharides, with no low molecular weight (LMW) carbohydrates present in the extract (data not shown), indicating that release of LMW carbohydrates as osmo-lytes had not taken place. Microscopy of samples stained for polysaccharides also confirmed the extraction of EPS. The efficient extraction by water is explained by the presence of cations that form bridges with the anionic groups of the polymers. It is proposed that water establishes an exchange of ions between the polymers and the water, thereby releasing the bonds of the polymer to the cell or other particles (Sutherland, 1980). It is likely that this does not happen to the same extent with NaCl, considering the high concentration of NaCl already present in seawater medium. Efficient extraction with water was also observed by Underwood *et al.* (1995) on carbohydrates extracted from intertidal sediments, al-though extractions of freeze-dried sediment or fresh cells may not be comparable.

The EPS of both species consisted mainly of carbo-hydrates, but some protein was present as well. Analysis revealed that considerable amounts of acid sugars were present in the form of uronic acids and sulphated sugars. This was confirmed by chemical analysis as well as by staining with Alcian Blue and subsequent microscopic examination. Attached EPS contained more uronic acids and sulphated sugars than non-attached EPS. This fraction was most closely associated with the cells. This is in accordance with the idea that attached EPS adheres to the outer cell surface through ion bridges by acid groups (Fletcher & Floodgate, 1973). The amounts of uronic acids, sulphated sugars and protein were comparable to those found for EPS isolated from other diatom species, such as *N. subinflata* (Bhosle *et al.*, 1995). This implies that these compounds are an integral part of diatom EPS fulfilling a specific function, for instance in adhesion to a substratum. The adherent properties of EPS could play a role in the locomotory mechanism (Wetherbee *et al.*, 1998). Some herbicides blocked EPS production by *Achnanthes longipes*, which resulted in loss of motility and adhesion (Wang *et al.*, 1997). Furthermore, Lind *et al.* (1997) showed that binding of an antibody to an extracellular proteoglycan inhibits adhesion and motility in *Stauroneis decipiens*. The binding of the antibody was competitively inhibited by uronic acids, also indicating that this component of EPS may be especially important in adhesion.

Monosaccharide composition of *C. closterium* and *N. salinarum* differed considerably but is generally com-parable to that in other diatom species (Darley, 1977). For instance, arabinose, if present at all, was found only in trace amounts. Glucose is probably mainly present as the β-1,3 glucan chrysolaminarin, which is known to be the

general storage carbohydrate in diatoms (Darley, 1977; Myklestad, 1988). Xylose and mannose are important components of the cell wall of diatoms. Mannose is also known as a compatible solute in diatoms (Paul, 1979).

Monosaccharides occurring exclusively in extracellular fractions were arabinose (both species) and galactose (*N. salinarum*). It is difficult to compare the monosaccharide composition of EPS with that of other diatom species. Mannose, rhamnose and galactose seemed to be the dominant sugars in many cases. There are only a few reported studies in which EPS consisted mainly of glucose. *Navicula subinflata* produced EPS that contained 94% glucose (Bhosle *et al.*, 1995). Capsular material from *Nitzschia angularis* consisted of glucose with trace amounts of rhamnose and xylose (Tokuda, 1969). It is clear from many studies that monosaccharide composition of diatoms varies widely among species (Hoagland *et al.*, 1993). Also, it is probable that monosaccharide composition will change with growth status. This remains to be investi-gated.

Non-attached EPS increased more or less at the same rate as protein content and cell number and hence did not show much change during the course of the experiment when expressed per unit protein or by cell number (Figs 3–6), although a slight increase was observed in *C. closterium* at the end of the log phase of growth. However, attached EPS showed a dramatic increase when the culture entered the stationary phase. It has been reported for many phototrophic microorganisms that stationary growth (generally induced by nutrient depletion) leads to production of intracellular (Hobson & Pariser, 1971; Darley, 1977; Myklestad, 1988) or extracellular carbo-hydrates (Lewin, 1955; Kroen & Rayburn, 1984; Myklestad *et al.*, 1989; Bhosle *et al.*, 1995). The secretion of non-attached EPS and attached EPS seemed to be under different metabolic controls. The possibility that non-attached EPS originated (partly) from cell lysis cannot be fully excluded, although it does not seem likely consider-ing the relatively low protein content of non-attached EPS. At this point it remains unclear whether the compositional difference between the two fractions is the consequence of a functional difference. The bulk of the polymers secreted remained attached to the cells. The non-attached polymers were present in much lower concentrations, and may represent some kind of a loss factor.

Anionic components such as uronic acids and SO_4^{2-} groups are of special interest because it is thought that these compounds are important for the adhesion proper-ties of EPS (Fletcher & Floodgate, 1973; Sutherland, 1980; Bertocchi *et al.*, 1990; Dade *et al.*, 1990). It is hypothesized that diatom biofilms on coastal mudflats greatly influence the stability and cohesiveness of these sediments (Holland *et al.*, 1974; Paterson, 1989; Decho, 1994; Yallop *et al.*, 1994; Sutherland *et al.*, 1998). In this investigation it was shown that the marine benthic diatoms *C. closterium* and *N. salinarum* produced considerable amounts of exo-polymers with SO_4^{2-} groups and uronic acids. Attached EPS in particular contained large amounts of these anionic

components. It is therefore proposed that the cell-associated EPS is important for sediment binding and stabilization. Since non-attached EPS was secreted into the medium it will probably be washed out from the sediment and consequently cannot contribute to sediment stability. Moreover, much more attached than non-attached EPS was produced. Secretion of EPS probably plays a role in adhesion to facilitate locomotion (Edgar & Pickett-Heaps, 1984; Lind *et al.*, 1997; Wang *et al.*, 1997). However, the results presented here and other reports (Bhosle *et al.*, 1995; Sutherland *et al.*, 1998) seem to indicate that, in addition, other factors such as growth phase control the amount of EPS produced. The role of nutrient depletion in growth and EPS secretion in cultures of *C. closterium* is currently under investigation. However, more experiments with field populations in their natural environment are necessary to confirm whether such factors also control EPS production in the field.

Acknowledgements

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