Dynamics in extracellular carbohydrate production by marine benthic diatoms

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Cover: diatom biofilms in the Biezelingse Ham mudflat (Westerschelde, the Netherlands), May 1998.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Intertidal mudflats are highly dynamic systems that are characterized by rapid changes in environmental variables. Both tidal and diurnal light cycles operate on mudflats, which may induce rapid fluctuations in light, salinity, temperature, water content, oxygen etc. In addition, sediment particles are frequently transported (eroded from the mudflat surface or deposited on top of the mudflat surface) under submerged conditions as a result of physical processes such as wave action and tidal currents. These processes result in the formation of a highly dynamic sediment-water interface.

Notwithstanding the extreme conditions that prevail in intertidal mudflats they represent highly productive systems that provide important feeding grounds for shorebirds and nursery grounds for fish. Benthic epipelic diatoms are the most important primary producers in intertidal mudflats (Admiraal 1984; Underwood & Kromkamp 1999). Diatoms are unicellular phototrophic algae that are characterized by a silica frustule enveloping the cell. The diatoms that live in sediments (called benthic diatoms) can be divided in epipsammic (attached to sediment) and epipelic (living freely on or in the sediment) species. Generally, epipsammic diatoms live in sandy sediments while epipelic diatoms dominate muddy sediments. Benthic epipelic diatoms supply organic matter to the benthic (Middelburg et al. 2000) as well as the planktonic foodweb (de Jonge & van Beusekom 1992, 1995) and may provide up to 50 % of total primary production in estuarine systems (Underwood & Kromkamp 1999). Epipelic diatoms excrete a considerable part of the photosynthetically fixed carbon as Extracellular Polymeric Substances (EPS) that mainly consist of carbohydrates (Hoagland et al. 1993; Staats et al. 1999). The diatoms become embedded in a matrix of EPS that is attached to the sediment. These structures are known as diatom mats or biofilms and are commonly found at the sediment surface of mudflats. By forming biofilms, epipelic diatoms create their own microenvironment that protects them from the rapidly changing conditions in intertidal mudflats (Decho 1994). Diatom biofilms also increase the stability of the sediment surface by increasing the erosion threshold (Paterson 1997, and references therein; Kornman & de Deckere 1998). In this way diatoms may have profound effects on the morphodynamics of mudflats (Dyer 1998) and influence sediment transport, potentially over the scale of whole estuaries (Frostick & McCave 1979).

Intertidal mudflats form an important component of coastal systems and play a vital role protecting shorelines from erosion and inundation by only modest defenses (Lee 2001).

An important characteristic of mudflats is the intense coupling of physical, biological, sedimentary and chemical processes that eventually determines the morphodynamics in these environments (Dyer 1998), (Fig. 1.1).

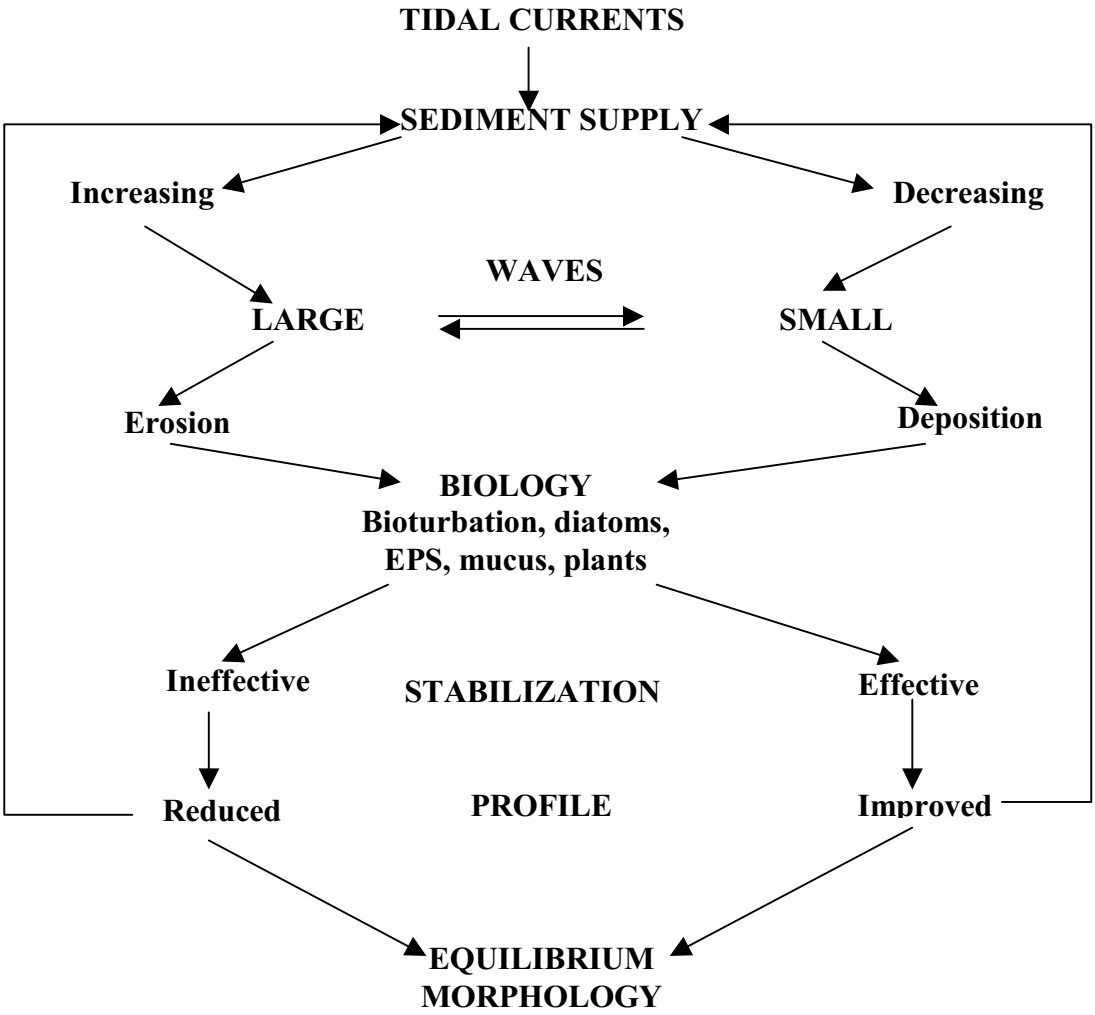

Figure 1.1. Schematic diagram of the interlinking of processes on intertidal mudflats.

Therefore, a comprehensive understanding of the functioning of intertidal mudflats necessitates an interdisciplinary approach in order to investigate biotic and abiotic processes as well as their interactions. This was provided by the EU projects INTRMUD (contract MAS3-CT95-0022) and CLIMEROD (contract MAS3-PL97-1605). In both projects, part of the research involved the investigation of the mechanisms and dynamics of extracellular carbohydrate excretion by benthic diatoms as well as their role in the biogenic stabilization of intertidal mudflats.

Excretion of extracellular carbohydrate by benthic epipelic diatoms

Many diatoms produce extracellular polysaccharides in the form of mucus, capsules, threads, pads, stalks or tubes (Darley 1977; Hoagland et al. 1993). Diatoms may excrete substantial amounts of photosynthetically fixed carbon in the form of extracellular carbohydrates. Estimates reported for epipelic diatoms range between 30-73 % of photosynthetate being excreted (Goto et al. 1999; Middelburg et al. 2000; Smith & Underwood 2000). Exopolysaccharides are excreted from a long narrow slit in the silica frustule called the raphe and are involved in gliding movement and substratum adhesion. Edgar & Picket-Heaps (1984) suggested that the role of EPS in gliding movement was to attach the diatom to a substratum. It was proposed that the EPS strands were then displaced parallel to the raphe as a result of contractions of microfilaments of the cytoskeleton, which would provide the force for gliding. Webster et al. (1985) identified the roles of actin- and tubuline-based microfilaments in gliding motility of the diatom *Amphora coffeaeformis.* The role of EPS was experimentally confirmed by Lind et al. (1997) by using antibodies to inhibit substratum adhesion and gliding of the diatom *Stauroneis decipiens*. In intertidal mudflats diatoms migrate in response to the light and tides (Paterson 1986; Pinkney & Zingmark 1991; Serôdio et al. 1997; Smith & Underwood 1998; Underwood & Smith 1998a). Since diatoms also migrate in dark, EPS excreted for this purpose should occur independent of the light. Indeed, Smith & Underwood (1998, 2000) observed dark production of EPS at the expense of intracellular carbohydrate in cultures as well as in intertidal mudflats.

Although it has not yet been quantified how much EPS are actually excreted during gliding, Edgar & Pickett-Heaps (1984) stated that movement would not require large quantities of mucilage and EPS-excretion would represent a low metabolic expense. Considering the copious amounts of EPS that are excreted by benthic diatoms this suggests that the production of extracellular carbohydrate may serve other purposes than motility only. Indeed, other workers (de Winder et al. 1999; Staats et al. 2000a) found that extracellular carbohydrate production was strictly light dependent and coupled to photosynthesis. It was suggested that this light dependent production of extracellular carbohydrate was caused by unbalanced growth as a result of nutrient depletion. This was supported by results from culture experiments (Lewin 1955; Myklestad & Haug 1972; Bhosle et al. 1995; Staats et al. 2000b) indicating that nutrient limitation (particularly nitrogen and phosphorous) enhanced exopolymer production. Also, Ruddy et al. (1998a) presented mass balance calculations that were in accordance with a mechanism of nutrient limitation. By coupling nitrogen limitation to carbohydrate production, Ruddy et al. (1998b) presented a model that accurately described short-term microphytobenthos and exopolymer dynamics in intertidal mudflat sediments.

Other variables may also affect the production of extracellular carbohydrates by marine diatoms. It was observed that adjustment of the salinity of the medium had an effect on growth and extracellular carbohydrate release in diatom cultures (Tokuda 1969; Allan et al.

Table 1.1. Monosaccharide distribution of extracellular polysaccharides of 6 different pennate diatoms (values indicate percentage of total). Fuc: fucose; Ara: arabinose; Rha: rhamnose; Rib: ribose; Gal: galactose; Glu: glucose; Man: mannose; Xyl: xylose. n.a.: not analyzed; -: not present; T: trace.

Species	reference	Fuc	Ara	Rha	Rib	Gal	Glu	Man	Xyl	Un-known
Nitzschia frustulum	Allan et al. (1972)	T	T	24	T	8	T	34	T	34
Nitzschia angularis		16	8	20	-	17	T	7	7	25
Navicula incerta		20	T	33	-	8	T	10	9	20
Navicula subinflata	Bhosle et al. (1995)	0.37	0.21	0.45	0.35	2.19	93.93	1.67	0.76	-
Cylindrotheca closterium non-attached EPS	Staats et al. (1999)	n.a.	0	14.7	n.a.	12.2	22.9	4.1	46.1	-
Cylindrotheca closterium attached EPS		n.a.	2.8	1.0	n.a.	2.2	82.5	7.6	3.9	-
Navicula salinarum non-attached EPS		n.a.	-	5.6	n.a.	19.1	41.6	13.8	20.2	-
Navicula salinarum attached EPS		n.a.	0.6	9.1	n.a.	4.3	85.1	0.5	0.3	-

1972). The effect of light levels and temperature have not yet been investigated in detail, but Staats et al. (2000a) observed that a certain amount of light was required in order to allow for the accumulation of exopolysaccharides. Besides quantitative information about exopolysaccharides excreted by the diatoms, knowledge about the chemical composition is essential in order to understand the mechanism(s) of EPS-excretion and their function in intertidal sediments. Few studies exist that analyzed the biochemical properties of EPS produced by pennate diatoms. These investigations indicated that 90.8 – 99.5 % of the EPS consisted of acidic polysaccharides, the remaining part being mainly protein (Bhosle et al. 1995; Staats et al. 1999). Analyses of the monosaccharide distribution of a number of species of pennate diatoms (Table 1.1) show that large differences exist in the composition of EPS excreted by various species of diatom (see also Lewin 1955; Tokuda 1969). Using pyrolysis-mass spectrometry, Smith & Underwood (2000) showed differences in the composition of EPS produced during the logarithmic and stationary phase in cultures of 5 epipelic diatoms. Hence, EPS-composition might depend on the physiological status of the organism. This was also found by Allan et al. (1972), who observed that the composition of EPS produced by *Nitzschia frustulum* were very different depending on whether the diatoms were cultivated

under high or low salinity. In addition, Wustman et al. (1997) observed that stalks produced by biofouling diatoms were composed of separate regions that were characterized by the presence of distinct types of EPS. Similarly, the application of sequential extraction procedures to obtain EPS from epipelic diatom cultures recovered two polysaccharide fractions that were largely different in composition (Staats et al. 1999, Table 1.1) and that were localized in different places.

Very little is known about the biochemical properties of polysaccharides in intertidal mudflats. Investigation of the exopolymer composition in intertidal sediments is complex due to methodological problems (recovery from a complex matrix, sample clean-up). Taylor et al. (1999) analyzed the monosaccharide distribution of a sample of colloidal and bulk carbohydrate from the Eden estuary (Scotland). It was found that the colloidal carbohydrates were rich in glucose (82 %) while this sugar was less abundant in the bulk carbohydrate fraction (37 %).

The role of biology in the morphodynamics of intertidal mudflats

It is well established that benthic diatoms are able to stabilize surface sediments of intertidal mudflats by means of the excretion of exopolysaccharides. The polysaccharides produced by benthic diatoms are anionic in nature and it is assumed that the negatively charged functional groups (uronic acids, sulfated sugars) play an important role cross linking the sugars and binding the sediment particles to form a biofilm (Decho 1994). Using Low Temperature Scanning Electron Microscopy (LTSEM), Paterson & Black (1999) showed that the matrix of diatom biofilms consists of a diffuse medium of polysaccharide strands that coat sediment particles, fill interstitial voids and form aggregates incorporating diatoms and particles. It is assumed that this results in the stabilization of the surface sediment layer, the area were diatoms are typically active (Paterson 1989, Sutherland et al. 1998). The stabilizing effect of EPS is probably not solely a result of increased cohesion between particles. The presence of diatom biofilms decreases the bottom roughness of the sediment bed, which reduces the surface shear for a given flow (Paterson & Black 1999, de Deckere et al. 2001) resulting in higher sediment stability. In addition, Ruddy et al. (1998a) emphasized the importance of the dynamic viscosity and the rheology of the sediment - water interface in the process of sediment erosion.

The interactions between factors that determine sediment stability in intertidal mudflats are complex and are comprised of both physical and biological components (Fig. 1.1). Yallop et al. (2000) proposed a relationship that related sediment stability to a combination of biological and physical variables (carbohydrates, chlorophyll *a* and water content). Amos et al. (1998) observed that variations in carbohydrate content at a station in the Humber mudflat (UK) had an impact on sediment stability up to 4 orders of magnitude greater

than equivalent variations in bulk density. However, at other stations the variations in sediment stability were a result of consolidation and dessication of the sediment and the biology did not appear to play an important role in the stabilization of the sediment. Similarly, Houwing (1999) studied temporal and spatial variations in erodibility of intertidal sediments and concluded that biology was of minor importance for the sediment stability. This indicates that although the potential of biogenic stabilization is high, it may vary to a large extend both on temporal and on spatial scales.

A vast amount of literature exists that investigates temporal and/or spatial dynamics in benthic biomass, extracellular carbohydrates, sediment stability or a combination of these over various temporal and spatial scales. For example, Taylor & Paterson (1998) investigated the distribution of extracellular carbohydrates present in bedforms (cm-m scale) over an emersion period. Other studies included whole mudflats (m-km scale) and sampled with weekly to monthly time intervals (Underwood & Paterson 1993a; Kornman & de Deckere 1998). In general, sediment stability appears to be high in the presence of diatom biofilms (i.e. high chlorophyll *a* and extracellular carbohydrate contents). However, when the erosion threshold is exceeded, the erosion rate and the sediment mass eroded appear to be higher in sediments with a diatom biofilm compared to uncolonized sediments (Widdows et al. 2000; Lucas et al. 2000).

Little is known about the significance of biogenic stabilization by microphytobenthos in the morphological development of intertidal areas. *In situ* treatments of diatom-inhabited sediments with biocides showed a rapid erosion of the sediment when the biota were killed (de Boer 1981; Daborn et al. 1993; Underwood & Paterson 1993a). A finer sediment fraction quickly winnowed out and the sediment bed formed ripples that are characteristic for non-cohesive sediments (Daborn et al. 1993). The magnitude of sediment transport to or from a mudflat, is determined by tidal currents (Postma 1961; Bell et al. 1997) and wind generated waves (Postma 1957; de Jonge & van Beusekom 1995; Bell et al. 1997). In intertidal mudflats it was observed that wind generated waves were important for resuspension of sediments (De Jonge & van Beusekom 1995). In addition, Christie & Dyer (1999) showed that offshore transport was correlated with the wave activity while onshore transport was determined predominantly by the maximum current speed. Erosion of several cm of sediment occurred during storms but this material was replenished in a few days under calm condition. Christie & Dyer (1999) did not study the effect of microphytobenthos, however, Daborn et al. (1993) reported that after a major disturbance diatoms were able to colonize the sediment over a period of a few days. Therefore, diatoms may play an important role by trapping sediment particles during the recovery of mudflats from erosion events. Furthermore, Frostick & McCave (1979) suggested that microphytobenthos were involved in trapping and binding up to 10^5 tonnes of sediment that were accreted on the banks of the Deben estuary (Suffolk, Great

Britain) in summer. This indicates that microphytobenthos may play an active role in sediment transport processes at the scale of estuaries.

In addition to microphytobenthos, other groups of organisms may directly or indirectly modify sediment processes. EPS-production has been observed in bacteria and the addition of bacterial EPS stabilized sandy sediments (Dade et al. 1990). EPS excretion was also reported for meio- and macrofauna (Probert 1984 and references therein). Therefore, these organisms may in principle enhance the sediment stability (Meadows et al. 1990; Mouritzen et al. 1998). In contrast, bioturbation activity by macrofauna increases the sediment erodibility (Rhoads et al. 1978; Widdows et al. 1998; Willows et al. 1998). De Deckere et al. (2001) pointed out that bioturbation increased the micro-topography of the sediment surface creating irregularities at the sediment surface. These microstructures act as initiators of erosion due to the micro-turbulence that is generated in the boundery layer. In addition, meio-and macrofauna may indirectly destabilize the sediment by grazing on microphytobenthos (Admiraal 1983; Gerdol & Hughes 1994). Furthermore, bacteria utilize extracellular products (van Duyl et al. 1999, 2000; Middelburg et al. 2000; Goto et al. 2001) and thereby alter the extracellular matrix. Few studies included the effect of different groups of organisms and trophic structure on the morphodynamics in intertidal mudflats. By doing so Daborn et al. (1993) found that interactions between the different trophic levels (microphytobenthos, macrofauna and migratory birds) strongly affected sediment properties on a tidal flat in the Bay of Fundy, Canada. Conversely, Probert (1984) hypothesized that natural disturbances (like storm events) and the initial (stabilizing) biogenic response are important in maintaining trophically mixed communities.

Outline of the thesis

The aim of this thesis was twofold. First, the dynamics in extracellular carbohydrates were studied at various temporal and spatial scales in order to identify the mechanisms (and relevant scales) of extracellular carbohydrate production by benthic diatoms. Secondly, detailed field surveys and laboratory experiments were carried out to identify the role of extracellular carbohydrate excretion by benthic diatoms in the morphodynamics of intertidal mudflats. For this purpose, both culture and field experiments were undertaken. *In situ* studies have the advantage that processes are investigated in sediments that are undisturbed (prior to sampling). However, other processes may obscure patterns in the production dynamics of extracellular carbohydrate by benthic diatoms. These include sediment – carbohydrate interactions, high background levels of carbohydrates, rapid turnover by heterotrophic consumers, wash out by the overlying water and production by organisms other than benthic diatoms. Laboratory experiments may largely avoid these problems. A consequence is however that the organisms are removed from their natural habitat and are grown in liquid

medium. Therefore, diatoms are not anymore exposed to the external forcing present at mudflats.

In addition to the quantification of extracellular carbohydrate levels, samples were regularly analyzed for their monosaccharide distributions as an indicator of the composition of the sugars. These measurements were carried out on culture as well as field samples and were considered to be a valuable additional tool to come to a better understanding of the mechanism(s) of exopolysaccharide production by benthic epipelic diatoms.

Axenic cultures of benthic diatoms were used to study the effect of alternating light-dark conditions on the production of EPS over a growth curve (chapter 2, 3). In chapter 2 the extraction procedures to recover EPS were evaluated using cultures of the benthic diatom *Cylindrotheca closterium.* The EPS fractions obtained with the sequential extraction procedure were characterized in terms of localization and temporal dynamics in EPS production. The production dynamics of extracellular and intracellular carbohydrates in cultures of the benthic diatoms *C. closterium* and *Nitzschia* sp. are described in Chapter 3. This study presents carbohydrate concentrations as well as the monosaccharide distributions of the EPS-fractions over a growth curve at the end of light and the end of dark periods. Chapter 4 describes the influence of different light conditions on the partitioning of photosynthetically fixed carbon between intra- and extracellular carbon pools. Furthermore, it provides a comparison between natural microphytobenthos and an axenic culture of *C. closterium*. Chapters 5 to 8 deal with *in situ* studies and describe dynamics in extracellular carbohydrates over various temporal and spatial scales. Chapter 5 presents the dynamics in microphytobenthos distribution and associated extracellular carbohydrates over 24 h periods (including emersion periods in the light and in the dark). Chapter 6 deals with the monthly temporal and spatial dynamics in biology, sedimentology and morphology in an intertidal mudflat. Chapters 7 and 8 focus on spatial patterns in distribution of extracellular carbohydrates. This includes a study investigating the effect of biogeomorphological features on the variations in microphytobenthos and extracellular carbohydrates in the Humber mudflat (UK), (chapter 7) and a comparison of the distribution and properties of extracellular carbohydrates in three west-European mudflats (chapter 8). Chapter 9 describes the sorption characteristics of two operationally defined EPS-fractions recovered from an intertidal mudflat. Furthermore, sediment slurries were enriched with these types of EPS and the effect of the EPS additions on the rheology of the sediment slurries was investigated. In chapter 10 the major findings in this thesis are discussed with respect to the mechanisms of EPS production and their dynamics and effects in intertidal mudflats.

CHAPTER 2

PHYSICAL CHARACTERIZATION AND DIEL DYNAMICS OF DIFFERENT FRACTIONS OF EXTRACELLULAR POLYSACCHARIDES IN AN AXENIC CULTURE OF A BENTHIC DIATOM

J.F.C. de Brouwer, K. Wolfstein & L.J.Stal

Abstract

The excretion of EPS by an axenic culture of the benthic diatom *Cylindrotheca closterium* was investigated. Two sequential extraction steps proved to be sufficient to remove the bulk of the EPS present. Soluble EPS was recovered by a simple centrifugation step and represented a fraction that was not or only loosely associated with diatom cells. For the extraction of bound EPS, different extraction procedures were compared. The best results were obtained using distilled water as extraction solvent (1 h, 30°C). The sugars that were recovered using this procedure were typically associated with aggregates of diatoms. Apart from the distinct differences in localization of the different types of EPS, also their temporal dynamics differed largely during an alternating light-dark cycle. Soluble EPS were continuously released into the medium with a rate of 1.6 $pg \cdot cell^{-1} \cdot day^{-1}$. In contrast, the production of bound EPS was highly light dependent. In the dark this bound EPS rapidly disappeared, probably as the result of its utilization by the diatoms.

Introduction

Benthic diatoms are an important component of microphytobenthos inhabiting intertidal mudflats (Underwood 1994). These organisms are able to excrete copious amounts of Extracellular Polymeric Substances (EPS) that are primarily composed of carbohydrates (Hoagland et al. 1993). These polymers play an important role in the ecology at intertidal mudflats. Likely, benthic diatoms themselves benefit from the excretion of EPS in various ways such as the ability to sequester toxic compounds, capturing of nutrients, protection against desiccation and motility (e.g. Decho 1990). For the ecosystem as a whole, excretion of EPS by benthic diatoms is important because it provides a food source for other organisms (van Duyl et al. 1999; Middelburg et al. 2000) and it stabilizes the sediment surface (Paterson 1997). The presence of diatom biofilms reduces shear stress on the bed (Paterson & Black 1999) and affects the rheology of the sediment water interface (Ruddy et al. 1998a) resulting in lower sediment erosion and capturing of fine grained material (chapter 6).

Mechanisms of EPS-excretion by benthic diatoms have been studied in intertidal sediments (Smith & Underwood 1998; Underwood & Smith 1998). However, the interpretation of data is hindered by sediment-EPS interactions, rapid turnover of EPS by heterotrophic consumers, high background levels of carbohydrates and wash out of carbohydrates by the overlying water. Controlled experiments to investigate the mechanisms of EPS excretion can be performed by using axenic cultures of benthic diatoms. This largely gets around the problems described above, although it should be kept in mind that the organisms are removed from their natural habitat and grown in liquid medium rather than on an intertidal mudflat.

Few studies have been published that investigated EPS-excretion by benthic diatoms in culture. A number of workers (Bhosle et al. 1995; Staats et al. 1999, 2000b; Smith & Underwood 2000) reported that enhanced EPS-excretion takes place under nutrient limitation. Furthermore, Staats et al. (2000a) showed that EPS-production was strictly light dependent. Smith & Underwood (2000) obtained similar results with soluble carbohydrates but observed that the EPS part of this fraction was also produced in the dark. Finally, Goto et al. (1999) compared EPS excretion in benthic diatoms and phytoplankton species. They concluded that production of EPS was more important in benthic diatoms while phytoplankton stored their fixed carbon predominantly as internal carbohydrates. Although among the different studies there is conformity of results on certain points, the interpretation may differ. Comparison of the results obtained in different studies would be useful to come to a better insight of the mechanisms that determine EPS production. However, this is hindered by the fact that procedures to extract EPS are different. Therefore, protocols that are used to extract EPS should be compared, not only in terms of recovery of EPS but also in terms of the biological and physiological meaning of the fractions analyzed.

In this study, a number of methods that are used for the isolation of EPS from cells in culture were compared and evaluated. Two operationally defined EPS fractions (soluble and bound EPS) were investigated in detail, both microscopically and in terms of temporal dynamics under an alternating light – dark cycle.

Material and methods

Culture conditions

The benthic diatom *Cylindrotheca closterium* was isolated from the Ems-Dollard (the Netherlands). Cultures were grown in 250 ml Erlenmeyers of which the bottom was covered with a layer of purified sea sand (Merck, Darmstadt, Germany). Cultures were established by inoculation of sterile medium (salinity 33 PSU) with an axenic strain of *C. closterium*. The composition of the medium is given in Table 2.1 and is essentially a modified F2-medium (Guillard & Ryther 1962) in which filtered seawater has been replaced by artificial seawater (Kester et al. 1967). Cultures were grown under a 12 : 12 h light-dark regime (~ 60 µmol photons·m^{-2}·s^{-1} incident light) at 20 °C. Cultures were regularly checked for contamination by microscopy and by plating on agar supplemented with glucose, yeast extract and Bacto-Peptone (Ferris & Hirsch 1991).

Carbohydrate analysis

Using a sequential extraction procedure, two operational fractions were analyzed that were termed soluble and bound carbohydrate according to the definitions formulated by Nielsen & Jahn (1999). Before sampling, the culture was homogenized by shaking by hand. An appropriate volume (2-10 ml) was taken for the extraction of the different carbohydrate fractions (n=3). The culture sample was centrifuged for 15 min at 3500×g. The supernatant yielded the soluble carbohydrate fraction. The pellet was used for comparison of different protocols to extract a carbohydrate fraction that is more tightly associated with the diatom cells. Extractants used were distilled water (milliQ), 4 mM EDTA (Goto et al. 1999) and 1.5 % NaCl. The extraction with distilled water was carried out at 30 °C while the extractions using 4 mM EDTA and 1.5% NaCl were done at 20°C. The cell suspensions were incubated for 1 h and subsequently centrifuged for 15 min at 3500×g. Carbohydrates were recovered from the supernatant. After the extraction of the bound carbohydrate fraction using the

Table 2.1. Composition of modified F2-medium for *C. closterium*.

Component	Concentration ($g \cdot l^{-1}$)	Component	Concentration ($g \cdot l^{-1}$)
NaCl	24.1	Trace metal mix:	
Na_2SO_4	32	$CuSO_4 \cdot 5\ H_2O$	$9.8 \cdot 10^{-6}$
$MgCl_2 \cdot 6\ H_2O$	8.7	$ZnSO_4 \cdot 7\ H_2O$	$22 \cdot 10^{-6}$
KCl	0.54	$CoCl_2 \cdot 6\ H_2O$	$10 \cdot 10^{-6}$
$CaCl_2 \cdot 2\ H_2O$	1.6	$MnCl_2 \cdot 4\ H_2O$	$18 \cdot 10^{-6}$
$NaHCO_3$	0.18	$Na_2MoO_4 \cdot 4\ H_2O$	$6.3 \cdot 10^{-6}$
KBr	0.039	Vitamine mix:	
NH_4Cl	0.0268	Biotin	$4 \cdot 10^{-8}$
H_3BO_3	0.011	Thiamine-HCl	$2 \cdot 10^{-5}$
$NaH_2PO_4 \cdot H_2O$	0.0069	Cyanocobalamin	$8 \cdot 10^{-7}$
$SrCl_2 \cdot 6\ H_2O$	0.010	Folic acid	$8 \cdot 10^{-8}$
$NaSi\ O_3 \cdot 9\ H_2O$	0.0426	Nicotinic acid	$4 \cdot 10^{-6}$
$Na_2Se\ O_3 \cdot 5\ H_2O$	$1.6 * 10^{-9}$	Thymine	$1.2 \cdot 10^{-4}$
Citrate mix:		Ca-d-pantothenate	$4 \cdot 10^{-6}$
$C_6H_8O_7 \cdot H_2O$	0.003	Inositol	$2 \cdot 10^{-4}$
Fe-NH_4-citrate	0.00036		

different extraction protocols, cells were checked for membrane integrity by microscope observations. For this purpose DIBAC (bis-(1,3-dibarbituric acid) trimethine oxonol, Molecular Probes, Oregon, USA) was used. DIBAC is a fluorescent marker of membrane potential and membrane integrity (Kisaalita & Bowen 1997; Arkhamar et al. 1998). Viable cells with a high membrane potential take up little of the dye, while cells with a low membrane potential (or compromised membranes) are permeable to DIBAC, and thus, labeled. To 0.99 ml of diatom suspension 10 µl of DIBAC-working solution was added to a final label concentration of 20 µM. Samples were incubated for 30 min under dim light conditions. Subsequently, the cells were filtered onto a polycarbonate filter (Ø 25 mm; 1.0 µm pore size). Cells were washed twice with sterile-filtered artificial seawater in order to remove excess of label. The diatom cells were resuspended in artificial seawater and examined under an epifluorescence microscope (Zeiss AXIOPLAN) equipped with a EX 480-500 nm excitation filter, a beamsplitter 510FT, and a longpass filter (LP520) in order to visualize autofluorescence of the chloroplasts as well as the fluorescent signal of the DIBAC label. The numbers of labeled and unlabeled cells were counted (counting a minimum number of 200 cells).

In both carbohydrate fractions a polymeric part, which is commonly termed the EPS-fraction (Underwood et al. 1995), was isolated by precipitation in cold ethanol (-20°C, 75 % final concentration). This EPS was allowed to precipitate overnight at -20°C. The mixture was centrifuged, the supernatant discarded and the pellet dried under a flow of N_2. The EPS was dissolved in 200 µl distilled water and carbohydrate was measured.

The glucan fraction was measured on the cell pellet that remained after the extraction of soluble and bound carbohydrates. To the pellet, 1 ml of 0.01 M H_2SO_4 was added and the mixture was stirred vigorously by vortex rotation every 30 min for a period of 2 h. Subsequently, the cell suspension was centrifuged for 15 min at 3500×g and sugars were analyzed in the supernatant (Myklestad & Haug 1972).

All carbohydrates were measured using the phenol-sulfuric acid assay (Dubois et al. 1956). To 200 µl sample, 200 µl phenol (5%, w/v in distilled water) and 1 ml concentrated H_2SO_4 was added. The mixture was shaken, incubated for 35 min and the absorbance was measured at 488 nm. Glucose was used as a standard.

Microscopy

Anionic carbohydrates were stained for microscopic observation using Alcian Blue (Crayton 1982). Cell suspensions of a culture in early stationary phase were dried on microscope glass slides. Uronic acids were stained using 0.1% (w/v) Alcian Blue in 0.5 M acetic acid (pH 2.5) while sulfated sugars were stained using 0.1% (w/v) Alcian Blue in 0.5 M HCl (pH 0.5). Samples were incubated for 30 min and carefully rinsed with water.

Temporal dynamics of soluble and bound EPS

The dynamics of the production of soluble and bound EPS by *C. closterium* was investigated during cultivation by taking samples at the end of the light and of the dark period. In order to be able to take these samples at the same time, two cultures were grown in parallel but at inversed light-dark cycles. In one culture the light period started at 9:00 h (Culture I), while in the other the dark period started at this time (Culture II). The cultures were sampled at 8:30 h. Cultures were first homogenized by shaking before a sample was taken. The light regimes that diatoms experienced differed slightly between the cultures that were sampled at the end of the light and the end of the dark period. This was because homogenization of the cultures resuspended the diatoms, which affected the light availability within the cultures.

In addition, EPS dynamics were investigated at a greater time resolution over a period of 3 days in the early and the late stationary phase of growth, by sampling up to three points during the course of a light or a dark period (n=3). Also for this experiment axenic cultures were grown in parallel with inversed light-dark cycles (Culture III, light period started at 9:00

h; Culture IV, light period started at 21:00 h). Soluble and bound EPS were isolated as described above (bound EPS was extracted for 1 h at 30°C using distilled water). In both experiments, the EPS-fractions were measured in triplicate and normalized to cell counts, which were determined by using a Coulter counter (Coulter Multisizer), operated with a 50 µm aperture tube.

Results

The comparison of different extraction procedures to isolate bound carbohydrates from *C. closterium* cultures is shown in Table 2.2. The highest concentrations of bound carbohydrate and bound EPS were obtained using 4 mM EDTA (16.1 ± 4.3 and 3.0 ± 0.1 $\mu g \cdot ml^{-1}$, respectively). The extraction with distilled water yielded 8.0 ± 2.0 and 1.5 ± 0.5 $\mu g \cdot ml^{-1}$ of bound carbohydrate and bound EPS, respectively, while this was 4.8 ± 0.4 and 1.2 ± 0.2 for the 1.5% NaCl extraction procedure. The glucan concentrations equalled 1 $\mu g \cdot ml^{-1}$ for the distilled water and the 1.5 % NaCl extraction procedures, while this was 40% less for the 4 mM EDTA-extraction. Staining of diatom cells using DIBAC as an indicator of membrane integrity showed that 11.7 ± 7.8 % of the cells were stained with DIBAC in the original culture. Centrifugation of the culture material to isolate the soluble EPS resulted in a similar percentage of cells incorporated with DIBAC (15.9 ± 19.5 %). All the extraction procedures applied to extract bound EPS resulted in increased numbers of DIBAC labeled cells. Extraction of bound sugars with 4 mM EDTA resulted in 100 % incorporation of DIBAC into the cells. For the extractions with distilled water and 1.5 % NaCl this was 43.6 ± 8.6 and 33.5 ± 12.5 %, respectively.

The localization of the soluble and bound carbohydrates was visualized microscopically using Alcian Blue to stain the anionic sugars (Fig. 2.1). These microscopic

Table 2.2. Comparison of the efficiency of bound carbohydrate extractions using different extraction solvents. Values in parenthesis indicate standard deviations (n=3).

Extraction solvent	Bound carbohydrate ($\mu g \cdot ml^{-1}$)	Bound EPS ($\mu g \cdot ml^{-1}$)	Glucans ($\mu g \cdot ml^{-1}$)	% DIBAC incorporated cells
distilled water	8.0 (2.0)	1.5 (0.5)	1.0 (0.3)	43.6 (8.6)
1.5 % NaCl	4.8 (0.4)	1.2 (0.2)	1.1 (0.1)	33.5 (12.5)
4 mM EDTA	16.1 (4.3)	3.0 (0.1)	0.6 (0.06)	100 (0.0)

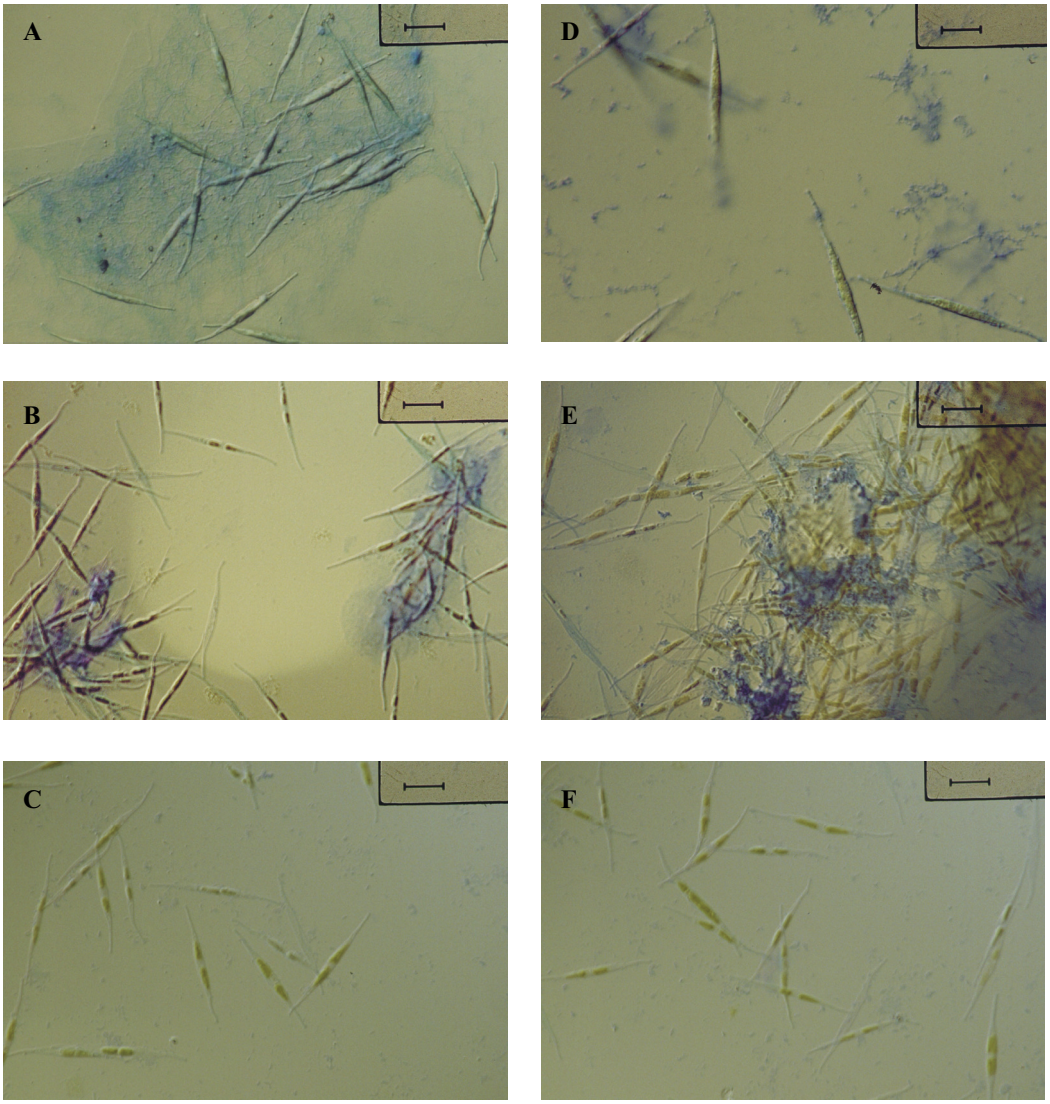

Figure 2.1. Differential Interference Contrast micrographs of *Cylindrotheca closterium* cultures stained with Alcian Blue (to visualize EPS) at different stages of the extraction procedure. (A); uronic acid stained before extraction procedure (B); uronic acid stained after extraction of soluble carbohydrates (C); uronic acid stained after extraction of bound carbohydrates (D); sulfated sugars stained before extraction procedure (E); sulfated sugars stained after extraction of soluble (F); sulfated sugars stained after extraction of bound carbohydrates. Bar markers in all panels except D: 17 µm; D: 10 µm.

observations revealed that both uronic acids and sulfated sugars were present in the EPS produced by *C. closterium*. The soluble carbohydrates were typically present as mucilage filling the space in between diatom cells (Fig. 2.1A) or as free flocs in the medium (Fig. 2.1D). Centrifugation of the culture removed these soluble carbohydrates. The so-called bound material that remained was associated with dense diatom aggregates (Fig. 2.1B, E) rather than with single cells. The extraction procedure applied (distilled water, 1 h, 30°C) removed the bulk of this bound fraction (Fig. 2.1C, F).

Growth curves for *C. closterium* initially increased exponentially in cell number and entered the stationary phase after 7 days (Fig. 2.2). Cell numbers in culture II were somewhat higher compared to culture I (Two way ANOVA, $F_{(1, 80)}$=66.04, p<0.001). Generation times were 22.1 and 20.1 h for culture I and II, respectively.

EPS concentrations were not detectable until day 5. From that day onwards, EPS contents (normalized to cell number) were analyzed regularly, both at the end of the light and at the end of the dark period (Fig. 2.3). Soluble EPS increased steadily over the period measured with a production rate of 1.6 pg·cell^{-1}·day^{-1}.

Production of bound EPS was strictly dependent on light, with the highest cell normalized production occurring in the exponential phase. In the dark, most of this EPS disappeared. The general patterns observed for the soluble and bound EPS-fractions were confirmed when the temporal dynamics were investigated in more detail (Fig. 2.4). In the early stationary phase, bound EPS increased rapidly during the light period (Fig. 2.4A) while the reverse was true during the dark period (Fig. 2.4B). The range over which the bound carbohydrate concentrations fluctuated was comparable for both cultures. However, overall there was a trend that bound EPS increased in culture III, while it decreased slightly in culture IV. The clear daily fluctuations that were found for the bound EPS were absent in the soluble

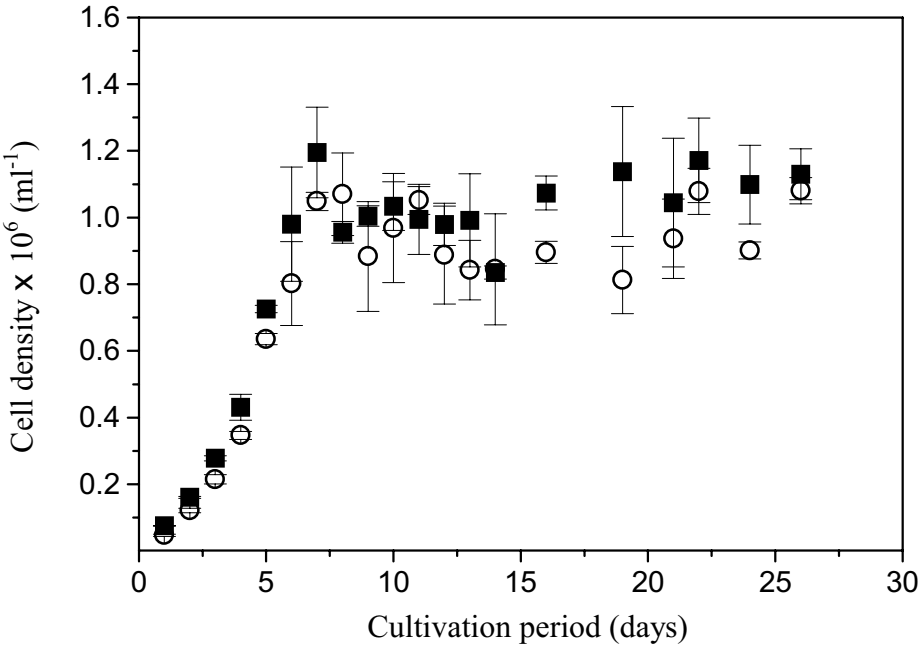

Figure 2.2. Growth curves for *C. closterium* in culture I (o)and culture II (■).Cultures were grown under a 12 : 12 h light-dark regime (~ 60 µmol photons·m^{-2}·s^{-1}) at 20 °C. Error bars indicate standard deviations (n=3).

EPS. In the soluble EPS-fraction a gradual increase was observed, indicating a continuous release of soluble EPS into the medium.

Discussion

Extraction of extracellular carbohydrates

In this study, two operationally defined carbohydrate fractions were analyzed from cultures of *C. closterium*. Soluble carbohydrates were recovered from the cultures by centrifugation (Decho & Lopez 1993; Staats et al. 1999; Smith & Underwood 2000). Staining of *C. closterium* with Alcian blue showed that centrifugation only removed a part of the EPS.

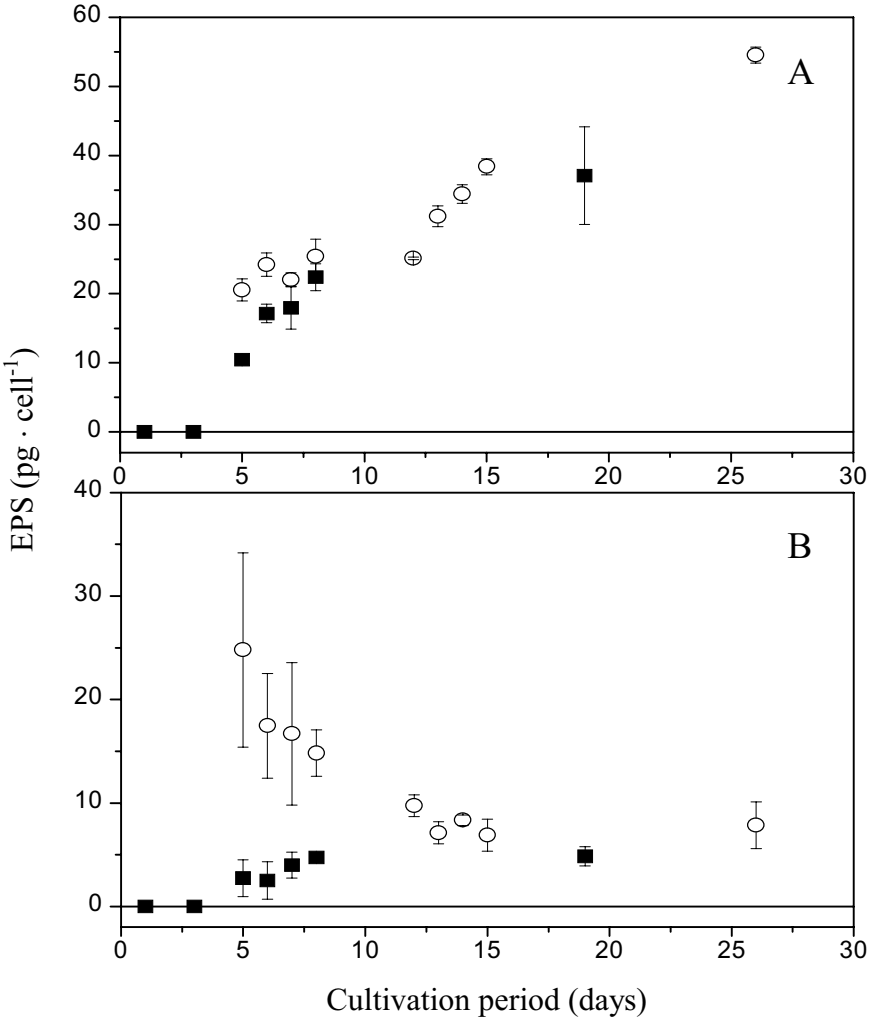

Figure 2.3. Cell-normalized amounts of soluble EPS (A) and bound EPS (B), sampled at the end of the light period (o) and the end of the dark period (■). Error bars indicate standard deviations (n=3).

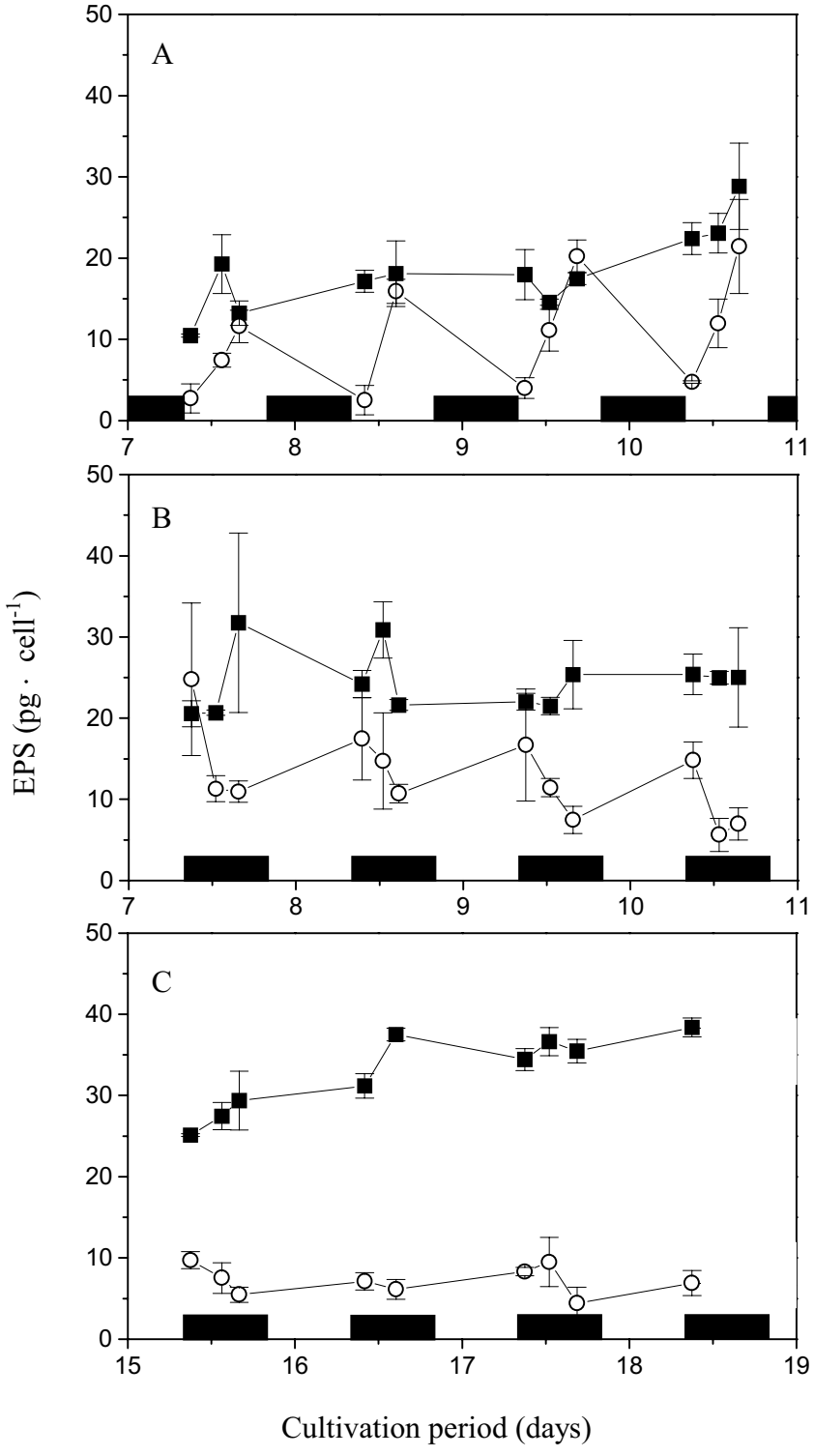

Figure 2.4. Cell-normalized amounts of soluble EPS (■) and bound EPS (o). (A); Samples taken during the light period in the early stationary phase, day 7-10 (culture III) (B); Samples taken during the dark period in the early stationary phase, day 7-10 (culture IV) (C); Samples taken during the dark period in the late stationary phase, day 15-18 (culture IV). Error bars indicate standard deviations (n=3).

distilled water extraction showed a higher recovery of bound carbohydrates, while concentrations of bound EPS were similar. The percentages of DIBAC labeled cells were not significantly different between the two extraction procedures and also glucan concentrations were similar. This either suggests that no intracellular sugars were released or that the amount of intracellular sugars that were released was similar between the two extraction methods. Therefore, distilled water was chosen for the extraction of bound carbohydrates. This method maximized the recovery of bound material using a readily available and reproducible extraction solvent, while contamination of EPS with intracellular sugars appeared to be at a level comparable to the 1.5 % NaCl extraction.

Microscopic observations using Alcian Blue showed that the two EPS fractions that were isolated by the sequential extraction procedure were localized differently. Centrifugation of the culture removed a part of the EPS that were not or only loosely associated with the diatoms. The remaining EPS were bound to diatom aggregates and the bulk of this material could be removed by extraction of the cell pellet with distilled water at 30 °C. Although for *C. closterium* extraction with distilled water at 30°C was sufficient to quantitatively isolate the EPS, properties of EPS excreted by diatoms may vary between diatom species. This may have an effect on the way this material is associated with the cells and on the extraction conditions that should be applied. For example, Lewin (1955) extracted EPS from *Navicula pelliculosa* using 20% (w/v) NaOH. This EPS was present as capsules surrounding single cells. In contrast, Bhosle et al. (1995) found that extraction with 1 M NaCl was sufficient to quantitatively remove bound EPS from the fouling diatom *Navicula subinflata*. Furthermore, EPS from the diatom *A. longipes* was present as stalks that could not be isolated quantitatively unless cells were extracted with 0.5 M $NaHCO_3$ at 95 °C (Wustman et al. 1997). These examples clearly show that the efficiency of the extraction of EPS may differ depending on the organism under investigation. Therefore, extraction protocols should be evaluated for quantitative removal of EPS prior to the actual measurements.

Temporal dynamics of the soluble and bound carbohydrate fractions

The two operationally defined fractions of EPS that were analyzed in this study showed distinct differences in localization of the polysaccharides as well as in the production dynamics under alternating light – dark conditions. Production of soluble EPS was evident from day 5 onwards and appeared to be independent of the light. This is in accordance with results of Smith & Underwood (2000) who also found that soluble EPS was produced both in the light and in the dark. These authors suggested that the excretion of this type of EPS played a role in the movement of benthic diatoms. Production of soluble EPS equaled 1.6 $pg \cdot cell^{-1} \cdot day^{-1}$. Smith & Underwood (2000) calculated EPS production rates for 5 species of benthic diatoms and found that production was on average 1 order of magnitude higher (18 $pg \cdot cell^{-}$

$^{1}\cdot day^{-1}$). The reasons for these differences in production rates are not clear at the moment but may be related to differences in the experimental setup between the two studies. Although Smith & Underwood (2000) used a lower incident irradiance, the light levels experienced by the diatoms may well have been higher in their replidishes (maximum volume of 5 ml) compared to our 250 ml Erlenmeyers. Furthermore, Smith & Underwood (2000) calculated maximum EPS production rates while our results are based on regression analysis.

Production of bound EPS occurred only in the light while in the dark most of this EPS disappeared. These findings are consistent with results of Hama & Handa (1992) who observed that in natural phytoplankton populations water-extractable polysaccharides decreased 40 % in the dark, with net production occurring exclusively in the light. Also in intertidal mudflats it was observed that EPS-production only occurred in the light while EPS contents decreased during periods of immersion or darkness (Staats et al. 2000a; chapter 5).

Various possibilities can be considered that might explain the observed decrease in bound EPS in the dark. For example, bound EPS may be transformed to soluble EPS, which would then explain the observed continuous increase in soluble EPS. However, over a large part of the growth curve the decrease in bound EPS was much larger that the concomitant increase in the soluble EPS fraction. It could also be argued that, in the dark, these HMW-sugars are degraded to polymers of a smaller MW that are not precipitated in cold ethanol. This would involve ecto- or exo-enzymes and hence represent a mechanism of breakdown of the EPS. Since the experiments were done with axenic cultures of diatoms, it excludes the possibility that bacteria were involved. Thus, the only possibility that remains is that the diatoms degraded the bound EPS themselves. It is known that benthic diatoms are capable of heterotrophic growth (e.g. Helleburst & Lewin 1977) and hydrolysis of EPS in cultures of *C. closterium* has been observed (Staats et al. 2000a; Smith & Underwood 2000). Thus, heterotrophic utilization of bound EPS might well have been possible. Also, Lancelot & Mathot (1985) showed that in *Phaeocystis pouchetti* colonies, freshly produced EPS were utilized in the dark. The EPS that were consumed in the dark were typically associated with cell aggregates. Similar results were obtained in this study, were a substantial amount of EPS (up to a maximum of 90 % of the EPS produced during the light period) associated with cell aggregates were removed in the dark. This suggests that EPS that are produced in the light could represent an integral part of the energy requirements in diatom aggregates (together with internal storage carbohydrates).

In cultures of benthic diatoms, the production of EPS associated with the organisms (Bhosle et al. 1995; Staats et al. 1999) as well as total EPS-production (Alcoverro et al. 2000; Staats et al. 2000b) has been attributed to nutrient depletion causing overflow metabolism. In this study, nutrient limitation was not likely in either of the EPS fractions analyzed. Production of soluble EPS was already observed during the exponential phase of growth and it continued at a constant rate during the stationary phase. Bound EPS increased somewhat in

the stationary phase in culture II (sampled at the end of the dark period), however, this was negligible when compared to the production during the light periods. In fact, during the course of growth, the amplitude of this diel fluctuation of bound EPS decreased (Fig. 2.3, 2.4C) and converged to a cellular content of about 5 pg. It is unlikely that this light dependent production was brought about by nutrient limitation because the highest production rates (on a cell-normalized basis) were observed in the exponential phase. Since the production of bound EPS was strictly light dependent, the decrease in the amplitude of the diel fluctuations can be explained by the lower average light intensity that diatoms experience when cell numbers and the size of the aggregates increase.

In conclusion, we have shown that the sequential extraction procedures applied by us yielded EPS-fractions that clearly differed in localization and in temporal dynamics. Soluble EPS was continuously released into the medium and was not or only loosely associated with diatoms. In contrast, EPS bound to the diatoms was produced exclusively in the light and to a large extend degraded in the dark. This study also showed that changes in EPS concentrations occur over a time scale of a few hours. These time scales should be considered when investigating the mechanisms that govern the excretion of EPS in benthic diatoms.

CHAPTER 3

DAILY FLUCTUATIONS OF EXOPOLYMERS IN CULTURES OF THE BENTHIC DIATOMS CYLINDROTHECA CLOSTERIUM AND NITZSCHIA SP. (BACILLARIOPHYCEAE)

J.F.C. de Brouwer & L.J. Stal

Abstract

Dynamics in the production of Extracellular Polymeric Substances (EPS) were investigated for the benthic diatoms *Cylindrotheca closterium* (Ehrenberg) and *Nitzschia* sp. By using axenic cultures, the effect of growth phase and alternating light – dark conditions were examined. Two EPS fractions were distinguished. One fraction of EPS (termed soluble EPS) was recovered from the culture supernatant and represented polysaccharides that were only loosely associated with the cells. The other fraction (termed bound EPS) was extracted from the cells using warm (30°C) water and represented EPS that was more closely associated with the diatom aggregates. Concentrations of EPS exceeded those of glucan concentrations throughout growth, indicating that EPS production was important in these organisms. Soluble and bound EPS revealed distinct differences in daily dynamics during the course of growth. Soluble EPS was produced continuously once cultures entered the stationary phase. During the stationary phase, chlorophyll *a*-normalized EPS-production rates equaled 6.4 and 3.4 d^{-1} for *C. closterium* and *Nitzschia* sp., respectively. In contrast, production of bound EPS occurred only in the light and was highest during the exponential phase. Up to 90 % of the attached EPS that was produced in the light was degraded during the subsequent dark period. The monosaccharide distribution of the EPS was analyzed by using HPLC and was constant during the course of the experiment. The soluble EPS consisted of high amounts of galactose and glucuronic acid, relative to rhamnose, glucose, xylose/mannose and galacturonic acid which were less abundant. In contrast, glucose was the dominant monosaccharide present in the bound EPS. These differences suggest that the production of the two operationally defined EPS fractions are under different metabolic controls and probably serve different functions for the cells.

Introduction

Benthic diatoms represent an important part of the microphytobenthos in intertidal mudflats (Underwood & Kromkamp 1999). A substantial part of the carbon fixed by these organisms is excreted as extracellular products (Goto et al. 1999; Middelburg et al. 2000; chapter 5) which are mainly composed of polymeric sugars (Hoagland et al. 1993; Staats et al. 1999). The excretion of Extracellular Polymeric Substances (EPS) by the organisms often results in the formation of diatom biofilms on the surface of the sediment of intertidal mudflats. The presence of these diatom biofilms increases the erosion threshold of the sediment (chapter 6; Sutherland et al. 1998; Paterson et al. 2000) and fine grained sediment particles that are deposited on the mudflat are bound to the EPS matrix (chapter 6; van de Koppel et al. 2001). Furthermore, EPS that is excreted by the diatoms may serve as a food source for bacteria (van Duyl et al. 1999; Goto et al. 2001) and macrofauna (Decho & Lopez 1993).

The mechanisms of EPS excretion in benthic diatoms are still poorly understood (Wetherbee et al. 1998). It is known that the excretion of EPS is involved in migration of diatoms (Edgar & Pickett-Heaps 1984; Lind et al. 1997). Since diatoms migrate also in the dark, EPS excreted for this purpose must occur independent of light. Indeed, Smith & Underwood (1998, 2000) observed dark production of EPS on the expense of intracellular storage carbohydrate in cultures as well as in natural mudflats. Similar observations were made by van Duyl et al. (1999) who observed that in a mudflat carbohydrate concentrations increased in the dark. However, other workers found that in intertidal mudflats excretion of EPS was coupled to photosynthesis (Staats et al. 2000a; chapter 5), while no production was observed in the dark. Finally, culture experiments showed that enhanced production of EPS occurred under nutrient limited conditions suggesting overflow metabolism as a mechanism of EPS-excretion (Bhosle et al. 1995; Alcoverro et al. 2000; Staats et al. 2000b). These results indicate that production of EPS may take place in response to various conditions including light- and nutrient availability, emersion/immersion of intertidal sediments and the physiological status of the organism. Moreover, the chemical and physical characteristics of the EPS may also vary in response to different conditions (Decho 1990; Wustman et al. 1997; Smith & Underwood 2000).

The aim of this study was to identify the effect of growth phase and alternating light-dark conditions on EPS-production by benthic diatoms. Therefore, the dynamics in EPS and intracellular storage sugars of two benthic diatoms cultivated under alternating light-dark conditions were investigated during the course of growth. The monosaccharide distribution of the EPS was measured over the growth curve in order to determine to what extent the EPS-composition varied over the course of the experiment.

Material and methods

Organisms and culture conditions

Cylindrotheca closterium was isolated from the Ems Dollard (the Netherlands) and provided by N. Staats (University of Amsterdam, the Netherlands). *Nitzschia* sp. was isolated from the Marennes-Oléron mudflat (France). Axenic cultures were cultivated at 20 °C in either 250 ml or 5 l Erlenmeyer flasks containing some fine sand (Merck, Darmstadt) that covered the bottom. The strains were grown in modified F2-medium with a salinity of 30 PSU (Table 2.1). Cultures were grown under a 12 : 12 h light-dark regime (ca 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ incident light, using white fluorescent lamps). Before and after the experiments cultures were checked for contaminating bacteria on agar plates and microscopically by Cybr green staining (Molecular Probes, Oregon, USA) or phase contrast microscopy.

Experimental design

The effects of daily variations of light and darkness and of growth phase were studied for *Cylindrotheca closterium* and *Nitzschia* sp. In order to enable sampling at the end of the dark and the end of the light period over a period of three weeks, two cultures were grown in parallel. In one of the cultures the light period started at 8:30 h, while in the other culture the dark period started at this time. The cultures were shaken at 20:00 h. Both cultures were sampled daily at 8:00 h. Prior to sampling, the cultures were homogenized by shaking and 200 ml were sampled for further analyses. This volume of culture material was sub-sampled and analyzed for chlorophyll *a*, soluble EPS, bound EPS, glucans and residual sugars (n=3). The EPS was analyzed for the monosaccharide distribution.

Isolation and analysis of carbohydrate fractions

Carbohydrates were measured in four different fractions using the phenol/H_2SO_4-assay (Dubois et al. 1956). The procedure that was used for the fractionation of carbohydrates is shown schematically in Fig. 3.1. Soluble EPS was obtained by centrifuging 10 ml of culture at 3500×g for 15 min at room temperature. The supernatant was transferred to a centrifuge tube containing 30 ml cold ethanol (96%) and the EPS was allowed to precipitate overnight at –20°C. After centrifugation (15 min at 3500×g), the EPS-pellet was dried under a flow of nitrogen and subsequently resuspended in 300 µl of distilled water. A volume of 200 µl was used for analysis of carbohydrate. Bound EPS was extracted by resuspending the culture pellet in 2 ml of distilled water. The cell suspension was thoroughly stirred and incubated for 1 h at 30°C. After centrifugation at 3500×g for 15 min the EPS was isolated and analyzed as

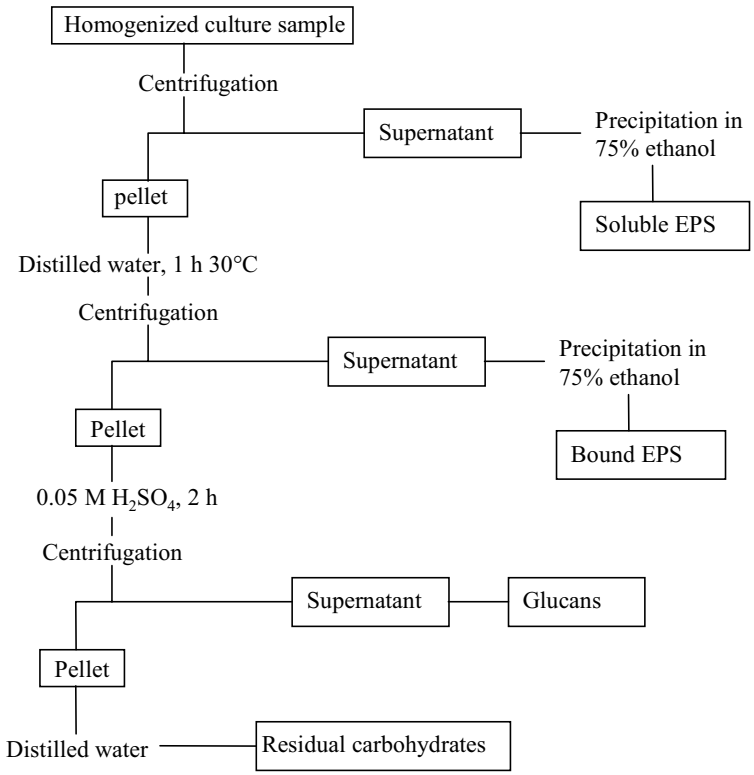

Figure 3.1. Flow chart of the fractionation of intra- and extracellular carbohydrate fractions.

described above. Glucans were isolated by using the procedures developed by Myklestad et al. (1972). Glucans were extracted by adding 1 ml 0.05 M H_2SO_4 to the cell pellet. The suspension was mixed every 30 min for a period of 2 hours. After centrifugation (15 min, 3500×g), 200 µl of the supernatant was used for carbohydrate analysis. For determination of the residual carbohydrates the cell pellet was resuspended in 400 µl distilled water. A volume of 200 µl of this suspension was used for analysis. This residual carbohydrate fraction consisted mainly of cellular carbohydrate (Smith & Underwood 2000).

Chlorophyll a-analysis

Chlorophyll *a* was taken as a measure of diatom biomass. For its determination, 10-30 ml of homogenized culture was filtered over a GF-F filter and stored at –80°C until further processing (n=3). Subsequently, the samples were freeze dried and extracted with 90 : 10 methanol : NH_4Ac for 1 h. During extraction the samples were sonicated for 1h at 0°C. Extracts were analyzed by HPLC according to Barranguet et al. (1997).

Monosaccharide composition

Soluble and bound EPS were analyzed for the monosaccharide composition of the polymers following the methods described in chapter 6. Briefly, freeze dried EPS samples were methanolized in 0.5 ml 2 M HCl in methanol (16 h at 85°C) and subsequently hydrolyzed to monosaccharides with 2 M trifluoro-acetic acid (1 h at 121°C). The monosaccharides were analyzed by HPLC equipped with a Carbopack-1 column (Dionex) and a Pulse Amperometric Detector (PAD, Dionex).

Statistical analysis

A two way ANOVA design was used to test the effects of cultivation period and light or dark period on the chlorophyll *a* and EPS concentrations. Replicate measurements were performed by taking subsamples from a single culture and are therefore pseudoreplicates. This means that in this statistical design it is not possible to discriminate between variation caused by the effect of light-darkness and the effect of the cultures itself. However, 24 h experiments with *C. closterium* that were conducted during the early and late stationary phase showed that similar fluctuations in EPS occurred as observed in this study (chapter 2). In addition, the growth curves for *C. closterium* and *Nitzschia* sp. were not significantly different between the cultures sampled at the end of the dark and the end of the light period (Fig. 3.2). From these observations we conclude that the cultures did not contribute substantially to the variation and that the variation was primarily caused be the light-dark effect. Statistical analysis for *Nitzschia* sp. were performed on log(n) transformed data. All statistical analyses were performed using the Statistica 5.1 package.

Results

Growth curves for *C. closterium* and *Nitzschia* sp. are shown in Fig. 3.2. *C. closterium* showed 2 days of lag phase and after a period of exponential growth, the cultures entered the stationary phase at day 5. During this phase, chlorophyll *a* concentrations slowly decreased. *Nitzschia* sp. cultures were in the lag phase during the first 3 days. The exponential phase lasted until day 8 after which a gradual increase in chlorophyll *a* was observed.

EPS-concentrations were measurable one day after chlorophyll *a* was detected. Soluble EPS levels increased from day 6 and day 9 onwards for *C. closterium* and *Nitzschia* sp., respectively (Fig. 3.3A, 3.4A). In both cultures, no significant differences were observed between samples taken at the end of the dark or light period, indicating that soluble EPS was continuously released into the medium. Soluble EPS normalized to chlorophyll *a* increased

linearly in the stationary phase (Fig. 3.3C, 3.4C) and production rates equaled 6.4 and 3.4 d^{-1} for *C. closterium* and *Nitzschia* sp., respectively. Bound EPS concentrations were significantly higher at the end of the light period when compared to the end of the dark period ($p<0.001$ for *C. closterium* and *Nitzschia* sp). This indicates that net production of bound EPS occurred in the light while part of this material disappeared in the dark (Fig. 3.3B, 3.4B). For both diatom cultures, the highest production of bound EPS was observed in the exponential phase and slowly decreased during the course of the experiment. For *C. closterium* there was a steady increase in EPS-concentrations at the end of the dark period. For samples taken at the end of the light period, concentrations initially decreased and thereafter leveled off at a concentration of 8 $\mu g \cdot ml^{-1}$ at the end of the experiment (Fig. 3.3B). Bound EPS concentrations in *Nizschia* sp. increased in the exponential phase and declined after a cultivation period of 2 weeks (Fig. 3.4B). Chlorophyll-normalized bound EPS-contents showed similar patterns (Fig. 3.3D, 3.4D).

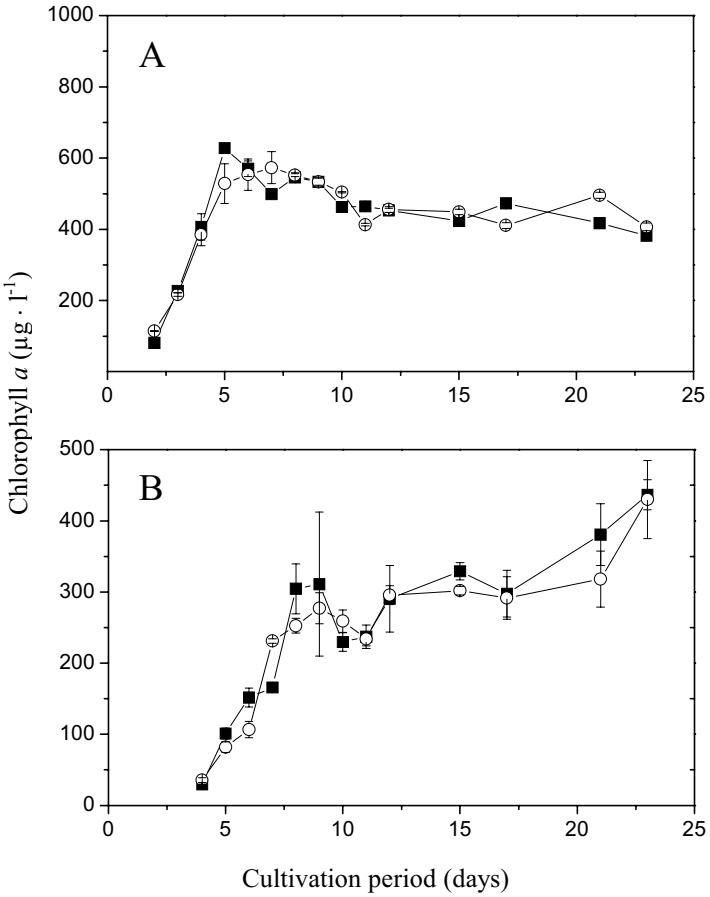

Figure 3.2. Chlorophyll *a* concentrations sampled at the end of the dark period (■) and the end of the light period (o) in axenic cultures of (A) *C. closterium* and (B) *Nitzschia* sp. Data points indicate mean values ± standard deviations (n=3).

Glucan and residual sugar concentrations and contents for *C. closterium* and *Nitzschia* sp. are shown in Fig. 3.5 and 3.6, respectively. Both glucans and residual sugars increased over the cultivation period. For *C. closterium* as well as *Nitzschia* sp., diel periodicity was observed for the glucans (Fig. 3.5A, 3.6A, $p<0.001$) but not for the residual carbohydrates (Fig. 3.5B, 3.6B). The production of glucans during the light period was compared to the production of bound EPS (Fig. 3.7). At the start of the experiment production of bound EPS exceeded that of the glucans. However, production of carbohydrates in both fractions changed with time. The amount of bound EPS decreased while glucans increased, leading to a higher production of the latter after a period of 10 and 16 days for *C. closterium* and *Nitzschia* sp., respectively.

Monosaccharide distribution was determined daily for both diatom species at the end of the light and the end of the dark period. The monosaccharide distribution of the different EPS-fractions hardly changed during cultivation and therefore the data were averaged. The results for *C. closterium* and *Nitzschia* sp. are shown in Table 3.1 and 3.2, respectively. Fucose, arabinose and glucose-amine were not present in detectable amounts in any of the EPS fractions measured. In both diatom species the monosacharide distribution of the two

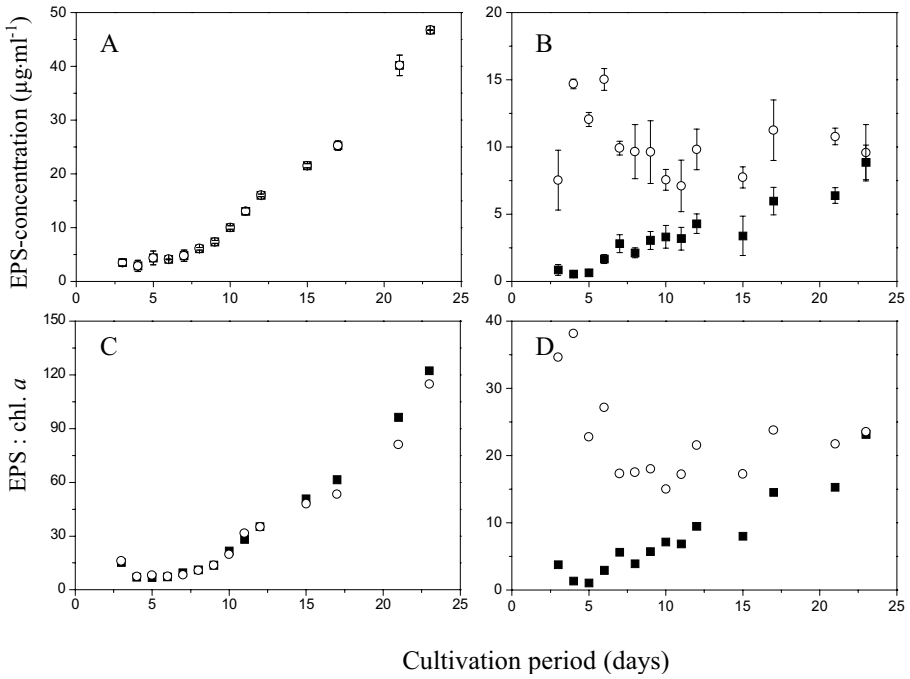

Figure 3.3. Soluble and bound EPS in axenic cultures of *C. closterium* sampled at the end of the dark period (■) and the end of the light period (o). (A) soluble EPS concentrations, (B) bound EPS concentrations, (C) soluble EPS contents and (D) bound EPS contents. Data points indicate mean values and error bars represent standard deviations (n=3).

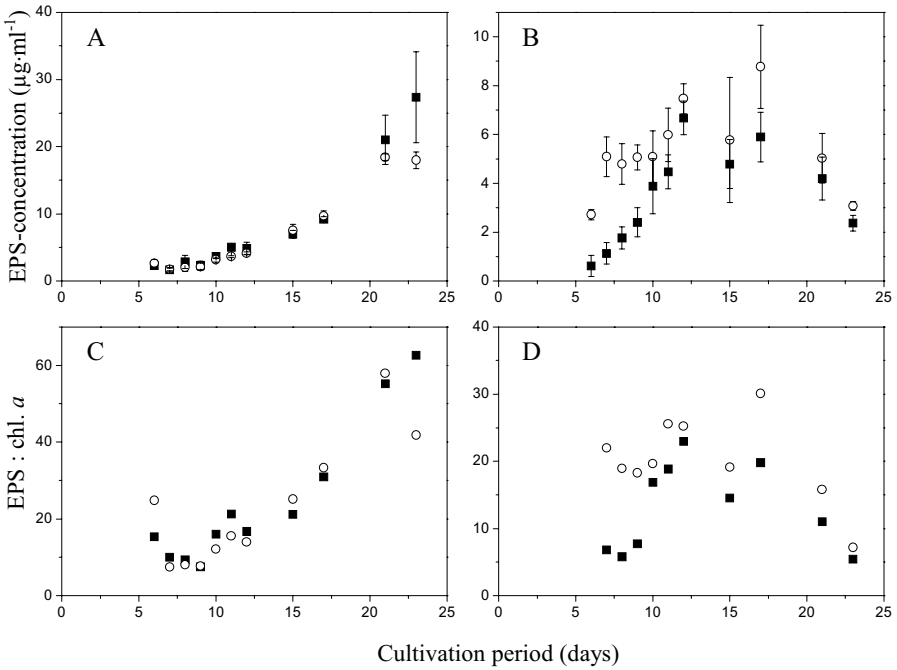

Figure 3.4. Soluble and bound EPS in axenic cultures of *Nitzschia* sp. sampled at the end of the dark period (■) and the end of the light period (o). (A) soluble EPS concentrations, (B) bound EPS concentrations, (C) soluble EPS contents and (D) bound EPS contents. Data points indicate mean values and error bars represent standard deviations (n=3).

EPS fractions revealed large differences. Soluble EPS typically consisted of high relative amounts of glucuronic acid, galactose and xylose/mannose while rhamnose, glucose and galacturonic acid were less abundant. For both diatom species, the monosaccharide distribution of soluble EPS sampled at the end of the dark period was similar to that sampled at the end of the light period. Compared to *Nitzschia* sp., *C. closterium* was somewhat enriched in galactose while rhamnose was less abundant. In the bound EPS, the most abundant monosaccharide present was glucose. During the light period the contribution of glucose increased from 55.3 ± 7.6 % to 72.0 ± 5.8 % for *C. closterium* and from 69.8 ± 10.1 % to 81.7 ± 8.0 % for *Nitzschia* sp. The increase in glucose was accompanied by a concomitant decrease in relative abundance of all other monosaccharides present. The monosaccharide distribution of the bound EPS that was produced in the light was calculated using the bound EPS concentrations and the relative monosaccharide distributions. *C. closterium* produced polysaccharides that were rich in glucose (81.8 ± 6.9 %). Similar calculations for *Nitzscha* sp. yielded negative contributions of several of the monosaccharides present and therefore it was not possible to obtain an accurate estimate of the glucose content in these polymers. However, the increase in relative abundance of glucose in the bound EPS

in the light indicated that the produced polysaccharides were also particularly rich in this monosaccharide.

Discussion

The experiments that were conducted in this study showed that the operational distinction between soluble and bound EPS was supported by the differences in composition and the dynamics of production. In the exponential phase, production of bound EPS was dominant, while in the stationary phase production of soluble EPS exceeded that of bound EPS. This shows that the excretion of both types of EPS is under a different physiological control. Similar observations were made by Smith & Underwood (2000), who found variations in the composition of EPS between the exponential and stationary phase of growth. Furthermore, the soluble and bound EPS were located at different sites in the cultures. Bound EPS was present associated with diatom aggregates (Staats et al. 1999; chapter 2) and could be extracted with warm (30°C) water, hence it was designated as 'bound' EPS. Soluble EPS

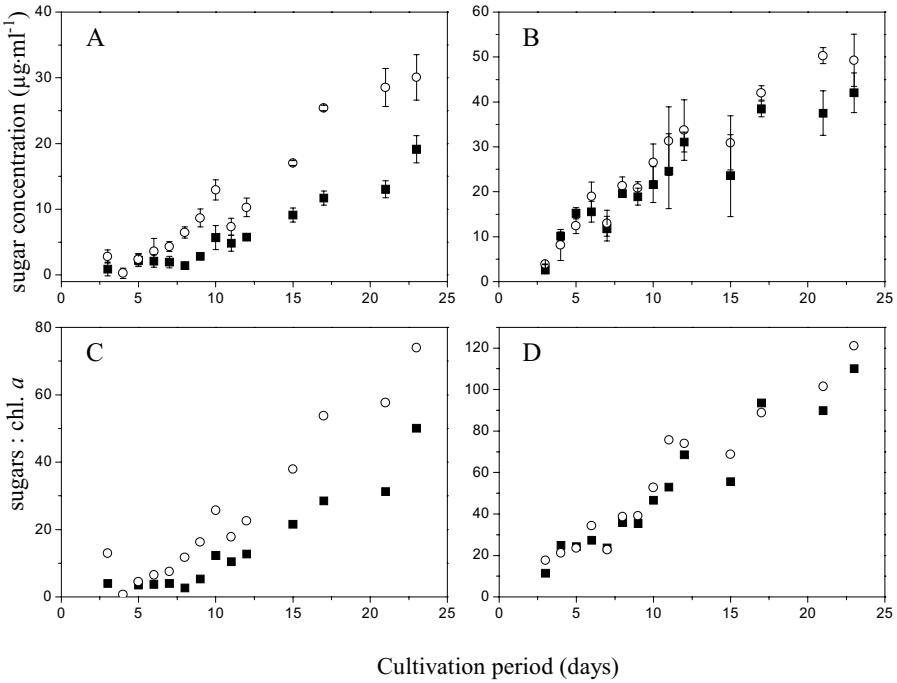

Figure 3.5. Glucans and residual carbohydrates in axenic cultures of *C. closterium* sampled at the end of the dark period (■) and the end of the light period (o). (A) glucan concentrations, (B) residual carbohydrate concentrations, (C)) glucan contents and (D) residual carbohydrate contents. Data points indicate mean values and error bars represent standard deviations (n=3).

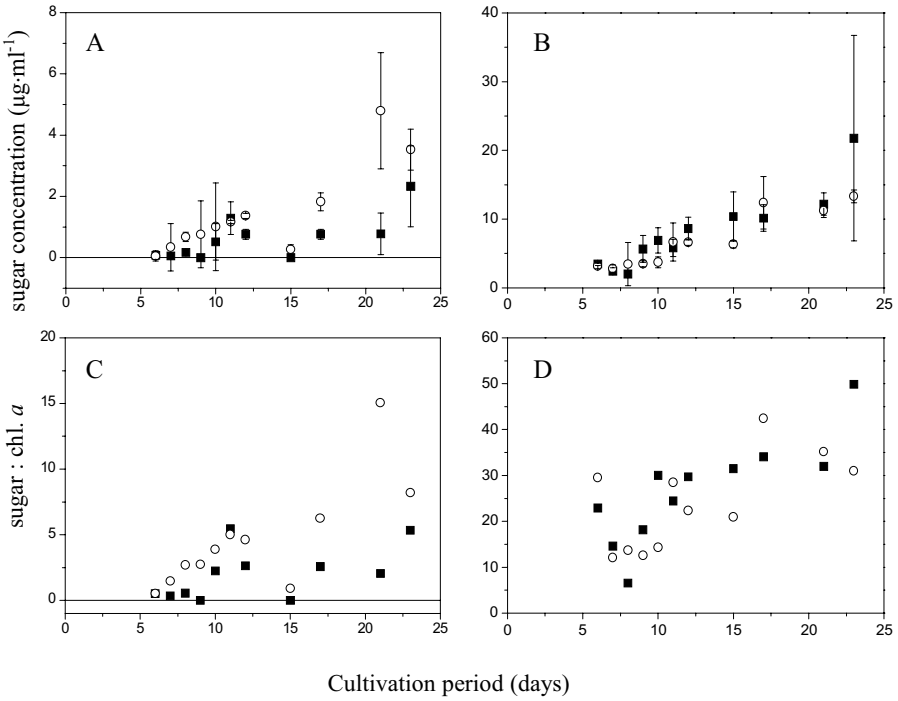

Figure 3.6. Glucans and residual carbohydrates in axenic cultures of *Nitzschia* sp. sampled at the end of the dark period (■) and the end of the light period (o). (A) glucan concentrations, (B) residual carbohydrate concentrations, (C) glucan contents and (D) residual carbohydrate contents. Data points indicate mean values and error bars represent standard deviations (n=3).

on the other hand was not associated with the diatom cells or aggregates and was found suspended in the medium, hence it was designated 'soluble' EPS. These differences in composition, localization and production strongly suggest that soluble and bound EPS serve different purposes and are probably under a different metabolic control.

The excretion of bound EPS was light dependent, while the major part of this material disappeared during the subsequent dark period. One explanation for the decrease in bound EPS during the dark period is that the EPS was dissolved in the medium and hence transferred to the soluble EPS fraction. However, evidence for dissolution of bound EPS was not found because we did not observe an increase in the amount of glucose in the soluble EPS during the dark period. Furthermore, the nighttime decrease in bound EPS was in general not balanced by the increase in soluble EPS during the same time period. Bacterial utilization of bound EPS was excluded because cultures were grown under axenic conditions. Therefore, we conclude that bound EPS served as an extracellular reserve product that was utilized by the diatoms during dark periods. This conclusion is supported by the fact that diatoms may be capable of heterotrophic growth (Hellebust & Lewin 1977) and that hydrolysis of EPS in axenic diatom cultures has been observed (Staats et al. 2000a; Smith & Underwood 2000). Moreover, Lancelot & Mathot (1985) observed that in the dark colonies of the Prymnesiophyceae

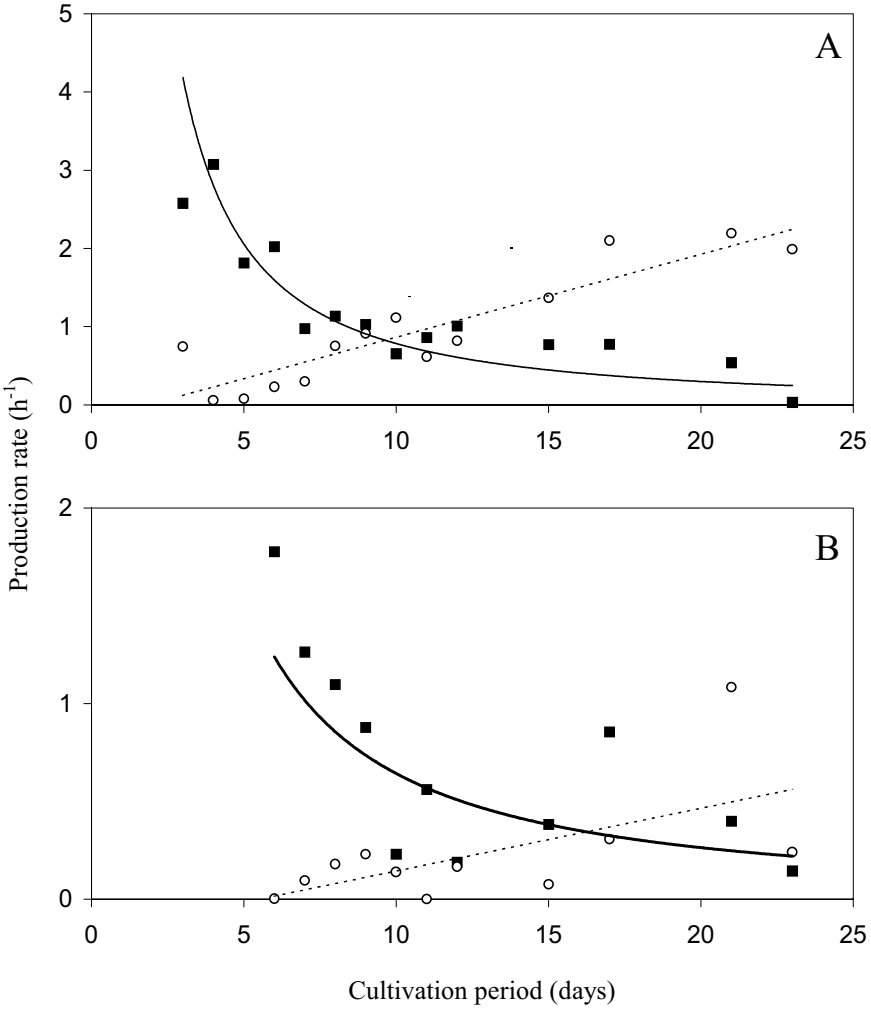

Figure 3.7. Daytime chlorophyll *a*-normalized production of bound EPS (■) and glucans (o) in axenic cultures of (A) *C. closterium* and (B) *Nitzschia* sp.

Phaeocystis pouchetti utilized the EPS that were produced in the light. These authors suggested that EPS (together with intracellular reserve products) act as energetic reserves during the dark period. This was consistent with our results, which showed comparable diel dynamics for bound EPS and glucans.

Release of EPS has been related to overflow metabolism caused by nutrient limitation (Myklestad & Haug 1972; Bhosle et al. 1995; Alcoverro et al. 2000; Staats et al. 2000b) or to migratory behavior of diatoms (Smith & Underwood 2000). For the bound EPS neither of the proposed mechanisms are likely to apply. Staats et al. (1999) suggested that the excretion of bound EPS was caused by overflow metabolism as a result of nutrient limitation (see also Bhosle et al. (1995)). However, in our hands the highest production of bound EPS was observed during exponential growth when nutrients were available in ample supply. Hence EPS-production as a result of nutrient limitation was not likely. Alternatively, the excretion of

Table 3.1. Relative distribution (weight%) of monosaccharides in EPS from *C. closterium*. Values are averages over the whole cultivation period. Values in parenthesis indicate standard deviations (n=14).

	Soluble EPS End dark period	Soluble EPS End light period	Bound EPS End dark period	Bound EPS End light period
Rhamnose	9.4 (1.8)	8.8 (1.3)	8.5 (1.5)	5.3 (2.0)
Galactose	29.2 (4.7)	28.1 (3.3)	14.3 (3.3)	8.3 (1.6)
Glucose	6.6 (4.7)	8.5 (4.9)	55.3 (7.6)	72.0 (5.8)
Xylose/mannose	18.4 (3.4)	17.2 (2.9)	11.1 (3.6)	6.2 (3.3)
Galacturonic acid	2.4 (3.8)	1.0 (2.3)	2.7 (2.4)	2.3 (1.4)
Glucuronic acid	33.9 (7.6)	36.4 (4.8)	8.1 (4.4)	5.8 (2.0)

Table 3.2. Relative distribution (weight%) of monosaccharides in EPS from *Nitzschia* sp. Values are averages over the whole cultivation period. Values in parenthesis indicate standard deviations (n=9).

	Soluble EPS End dark period	Soluble EPS End light period	Bound EPS End dark period	Bound EPS End light period
Rhamnose	11.6 (3.1)	14.0 (3.8)	5.8 (3.2)	3.7 (2.5)
Galactose	21.1 (4.3)	24.5 (6.0)	7.1 (2.9)	4.5 (2.0)
Glucose	8.2 (3.7)	11.0 (4.5)	69.8 (10.1)	81.7 (8.0)
Xylose/mannose	14.6 (3.2)	17.6 (4.5)	8.0 (4.4)	4.9 (2.1)
Galacturonic acid	2.7 (5.2)	2.9 (4.5)	1.6 (2.2)	0.0 (0.0)
Glucuronic acid	41.7 (1.0)	30.1 (13.0)	7.7 (2.9)	5.1 (1.7)

EPS connected to migration takes place in response to the light and tides (Serôdio et al. 1997; Smith & Underwood 1998), and should occur regularly during periods of darkness. Therefore, production of EPS for migratory purposes should be largely independent of light conditions. This was not in agreement with our observations that the production of bound EPS was strictly light dependent, while it was degraded during the dark period. The results shown in Fig. 3.7 hint to another mechanism that may explain the dynamics of the bound EPS fraction. Glucan production increased during the course of the experiment indicating that the capacity to store carbohydrate inside the diatom cells increased. At the same time the contribution of bound EPS decreased. Therefore, it is possible that the amount of carbohydrate that was excreted as bound EPS was directly connected to the capacity of the diatom cells to store reserve carbohydrate intracellularly. In addition, the analysis of the monosaccharide distribution of the bound EPS revealed a particularly high content of glucose in the polymers. Chrysolaminaran, which is the common storage glucan in benthic diatoms (Darley 1977)

consists mainly of glucose moieties. The resemblance of these glucans and extracellular carbohydrates in terms of their monosaccharide composition as well as the correspondence in production dynamics under alternating light dark conditions suggests that these fractions are closely related and may be under the same metabolic control.

The production of soluble EPS was evident during the stationary phase and continued in the light and in the dark. This continuous release of soluble EPS is in agreement with the observation of Smith & Underwood (2000) who found active production of EPS during periods of darkness. These authors suggested that the EPS played a role in the migration of diatoms and therefore production was independent of light conditions. Alternatively, EPS-production in the stationary phase has been attributed to unbalanced growth in phytoplankton, (Myklestad & Haug 1972), benthic diatoms (Staats et al. 1999) and biofouling diatoms (Bhosle et al. 1995). The production dynamics of soluble EPS that were observed by us were in accordance with the model of Ruddy et al. (1998b). These authors suggested that migration of diatoms occurred under conditions of light or nutrient limitation in order to improve growth conditions. Furthermore, it was assumed that carbohydrate production occurred during periods of nutrient limitation, thereby coupling migration to EPS-production in the stationary phase. Thus, the observed production dynamics for the soluble EPS may be a consequence of enhanced diatom migration in order to attempt to optimize growth conditions (for example, to increase nutrient or light availability). In the dark, soluble EPS could only be synthesized at the expense of glucans and bound EPS. We calculated that for *C. closterium* and *Nitzschia* sp. respectively, 10-17 % and 18-22 % of the bound EPS and glucans were utilized for the production of soluble EPS.

Monosaccharide analysis of soluble EPS indicated that the structure was complex and contained a high amount of uronic acids (33-44 %). The complexity of the polymers could hint to a specific function of the EPS (e.g. motility or substratum adhesion) rather than being a product of unbalanced growth as seemed to be the case for the bound EPS. Indeed, Dade et al. (1990) showed a correlation between the amount of uronic acids present in bacterial EPS and the erosion threshold of sandy sediment, indicating that uronic acids may play a role in the binding of EPS to a substratum. In addition, Lind et al. (1997) were able to inhibit movement and adhesion of the diatom *Stauroneis decipiens* by binding an antibody to extracellular proteoglycans present on the cell surface and in the raphe. Uronic acids competitively inhibited binding of the antibody, which indicated that this component may play an important role in motility and adhesion.

The production dynamics of extracellular carbohydrates by benthic diatoms have also been investigated in intertidal mudflats (de Winder et al. 1999; Taylor et al. 1999; Staats et al. 2000a; chapter 5) and can therefore be compared to the results obtained in this study. It has frequently been observed that EPS production by natural communities of diatoms is light dependent (de Winder et al. 1999; Taylor et al. 1999; Staats et al. 2000a; chapter 5). The EPS

produced in the light disappeared during subsequent periods of tidal inundation which may have been due to heterotrophic utilization of the EPS (van Duyl et al. 1999; Middelburg et al. 2000; Goto et al. 2001) or to dissolvation of EPS into the overlying water column (Underwood & Smith 1998; de Winder et al. 1999). In addition, monosaccharide analyses of the EPS produced in the natural situation showed a high proportion of glucose (82-90 %), (Taylor et al. 1999; chapter 5). Hence, the excretion of EPS by diatoms in their natural habitat closely resembled the excretion of bound EPS in cultures, both in terms of production dynamics and in terms of the monosaccharide distribution. This would mean that the excretion of EPS in intertidal mudflats is probably not the result of diatom migration or nutrient limitation. This is in line with the observation of Staats (1999) that excretion of EPS is generally much higher than the amount that would theoretically be required for motility (Edgar & Pickett-Heaps 1984). Furthermore, Kromkamp et al. (1998) considered that nutrient limitation even in dense diatom mats was not likely because of the high nutrient values observed in the pore water of the surface sediment. Hence, EPS excretion may largely be explained to be the result of unbalanced growth, which occurs when the amount of carbon that is fixed as carbohydrate exceeds the capacity to store carbohydrate inside the cells (see discussion above).

In summary, it was shown that for the benthic diatoms *C. closterium* and *Nitzschia* sp two different types of EPS were excreted under different conditions. Over the full growth curve the contribution of extracellular polysaccharides (sum of soluble and bound EPS) was always higher than the contribution of glucans, which is in accordance with other workers who found that 40-70 % of fixed carbon was excreted as extracellular products whereas 7-30 % was present as internal storage compounds (Goto et al. 1999; Smith & Underwood 2000; chapter 4). The dynamics in production and the composition of the soluble and bound EPS were different. Bound EPS was produced in the light and consisted of a glucose rich polymer. Both dynamics and composition of this EPS resembled EPS production in the field under bloom conditions. Soluble EPS was typically a heteropolymer, rich in glucuronic acid. Dynamics and composition of this EPS-fraction suggests that it may be involved in diatom movement or substratum adhesion.

CHAPTER 4

BIOCHEMICAL PARTITIONING OF PHOTOSYNTHETICALLY FIXED CARBON BY BENTHIC DIATOMS DURING SHORT-TERM INCUBATIONS AT DIFFERENT IRRADIANCES

K. Wolfstein, J.F.C. de Brouwer & L.J. Stal

Abstract

The partitioning of photosynthetically fixed carbon into different fractions of intracellular and extracellular carbon pools by an axenic culture of *Cylindrotheca closterium* (Ehrenberg) and natural microphytobenthos was studied using short-term incubations with ^{14}C at different irradiances. Both in axenic cultures of *C. closterium* and in natural populations of microphytobenthos, excretion of photosynthetically fixed carbon was propertional to the rate of photosynthesis. Hence, excretion was directly dependent on the level of irradiance. During the incubations, about 70 and 75% of the fixed carbon, respectively, was excreted by the culture and the field samples. This excreted carbon was distinguished in two operationally defined fractions, which were termed soluble and bound carbon fractions. The percentage of excreted carbon was constant for the cultures over the range of different irradiances. The percentage of Extracellular Polymeric Substances (EPS) in the soluble and bound material as obtained by ethanol precipitation was constant over the range of irradiances that were applied. Subsamples of natural microphytobenthos were treated with an antibiotic cocktail in order to exclude bacterial activity, which resulted in unexpected higher values of incorporated carbon in all fractions.

Introduction

The benthic microalgal community inhabiting cohesive sediments often consists of a large number of epipelic diatoms (Underwood 1994; Underwood & Kromkamp 1999). These diatoms are known to excrete highly hydrated, carbohydrate-rich substances. During the stationary phase up to 50% of extracellular material may consist of Extracellular Polymeric Substances (EPS) (Smith & Underwood 2000). These exopolymers are of importance for these algae as they are involved in various processes such as capturing of nutrients, motility and protection against desiccation (Hoagland et al. 1993). They also play a role in sediment stabilization (Paterson 1989) and enter the food web as a source of organic matter for bacteria and grazers (Decho 1990; Smith & Underwood 1998; van Duyl et al. 1999). Due to the importance of these substances, several studies have focussed on their characterization, quantification and on the identification of the factors that control their production. These factors include the availability of nutrients (Sutherland et al. 1998; Staats et al. 2000b) and the effect of irradiance (Smith & Underwood 1998; Staats et al 2000a). A direct relationship between the production of exopolymers and oxygenic photosynthesis was found by Staats et al. (2000a). These authors did not investigate the effect of different light intensities on exopolymer production. However, different light intensities may have direct consequences on the amount of EPS produced by microphytobenthos during different seasons.

The objective of this study was to investigate the effect of different irradiances on the quantity of exopolymer production and on the short-term partitioning of the photosynthetically fixed carbon into different intra- and extracellular fractions. Results from laboratory cultures are difficult to translate to the field. Therefore, we applied similar methods to isolate the various carbon fractions in diatom cultures and in a natural microphytobenthic community, freshly collected from a nearby mudflat in the Westerschelde estuary (The Netherlands). In this way it was possible to compare the excretion of photosynthetically fixed carbon in cultures with the excretion in the field samples. Natural microphytobenthos contained bacteria while this was not the case in benthic diatom cultures. Bacteria may influence the dynamics of extracellular substances in various ways. Bacteria are able to excrete EPS themselves (Decho 1990) and may degrade exopolymers (Goto et al. 2001). Therefore, the effect of bacteria on the dynamics of extracellular substances was studied by using antibiotics to eliminate bacterial activity.

Material & Methods

Culture conditions and sampling

An axenic strain of *Cylindrotheca closterium* (Ehrenberg) was used for the experiments. Cells were grown at 15 °C in modified F2-medium (Table 2.1) at an incident photon irradiance of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by Philips 'TLD 15W/840' fluorescent tubes, supplied at a 12:12 h light:dark cycle. The algae were cultured in 1 l glass Erlenmeyer flasks in which the bottom was covered with purified seasand (Merck, Germany). The cultures were subsampled at early stationary growth phase (day 9 and 10, respectively). Prior to sampling the culture was gently shaken until all cells were suspended. Subsequently, the sand was allowed to settle after which a sample of the algal suspension was taken.

One day before the experiment started, the natural population of benthic diatoms was sampled by scraping off the surface layer of sediment from the sampling site "Biezelingse Ham" at the Westerschelde, The Netherlands (51°26'474''N, 3°55'506''E) at ebb tide. The sample was put in a jar, covered with three layers of lens tissue (Whatman) and incubated for several hours at an irradiance of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The motile algae migrated through the tissue to the surface and were harvested by removing the upper layer of tissue. The algae were suspended in modified F2-medium, and kept at 15 °C and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ until the experiments started. Subsamples of the culture and the field sample were stained with Sybr-green and microscopically studied in order to check axenity of the cultures and to count bacteria in the field sample.

Experimental set-up

At the start of each experiment, subsamples were taken for chlorophyll *a*-analyses (n=3). A volume of 15 ml of algal suspension was centrifuged for 10 min at 3500×g using a Sigma 4K15 centrifuge. Chlorophyll *a* was extracted from the cell pellet using 5 ml of 96 % aceton and the extract was subsequently measured with an Ultraspec 4000 spectrophotometer (Jeffrey & Humphrey 1975).

The effect of irradiance on the partitioning of photosynthetically fixed carbon *C. closterium* and natural microphytobenthos was investigated by means of short-term incubations (30 min) with ^{14}C. Three replicate measurements were performed for both the culture and the field samples. However, for technical reasons these were measured over three different days. For the incubations, 15 ml of algal suspension was transferred to the cuvette of a DW3 oxygen chamber (Hansatech). To this sample, 400 µl of $NaH^{14}CO_3$ (Amersham, final activity of 370 KBq per sample) was added. The suspension was well mixed by a magnetic stirrer situated at the bottom of the cuvette. The samples were kept in the dark for 5 min and

subsequently exposed to various light irradiances for a period of 30 min. The temperature was kept constant at 15±1 °C. Incubations were performed at 9 different irradiances (8, 26, 53, 65, 120, 228, 460, 1065 and 1765 $\mu mol \cdot m^{-2} \cdot s^{-1}$ incident light) which were applied in random order. Dark and poisoned (glutaraldehyde, 3 % final concentration;) controls were included in order to account for heterotrophic and passive/abiotic uptake. The incubations were stopped by the addition of glutaraldehyde (3 % final concentration). After applying the different extraction steps (described below), non-incorporated carbon was removed by adding 100 µl concentrated HCl. Subsequently, Packard scintillation cocktail was added and ^{14}C incorporation was measured with a Packard Tri-Carb 2300 TR scintillation counter, including quench correction. The concentration of dissolved inorganic carbon present in the medium was determined by potentiometric titration.

For each incubation, incident and outcoming irradiance was measured with a LICOR quantum sensor Q19736 connected to a LI-1000 data logger. Mean values of light irradiance in the cuvet (E) were calculated according to the formula of van Liere & Walsby (1982):

$$E = (E_0 - E_z / (\ln E_0 - \ln E_z) \quad (1)$$

E_0 is the incident irradiance and E_z is the irradiance coming out of the cuvette. Photosynthetic efficiency (α^B), maximum photosynthetic rate (P^B_{max}) and the light saturation parameter (E_k) were calculated using the fit of Walsby (1997). The superscript "B" indicates that photosynthetic parameters were normalized to chlorophyll *a*.

Fractionation of the different pools of carbon

A flow chart of the isolation of the fractions of total fixed carbon, intracellular carbon, glucans, soluble carbon and EPS, and bound carbon and EPS is shown in Fig. 4.1. After incubation with $NaH^{14}CO_3$ the algal suspension was divided into two subsamples that were centrifuged for 15 min at 5310×g. The supernatant contained the soluble extracellular carbon fraction. Subsequently, the cell pellet was extracted with distilled water (1h, 30°C) in order to obtain the bound extracellular carbon fraction. The high molecular weight parts (termed EPS) of the soluble and bound carbon fractions were obtained by overnight precipitation in cold ethanol (final amount of 75 %) at –20°C. The cell pellet of the first subsample that remained after extraction of the soluble and bound carbon fractions was extracted in 0.1 N H_2SO_4 for a period of 2 h in order to analyze storage glucans (according to Smith & Underwood (1998)). The cell pellet of the second subsample (after extraction of soluble and bound carbon) was directly analyzed for the amount of incorporated ^{14}C. This fraction was termed the intracellular carbon fraction.

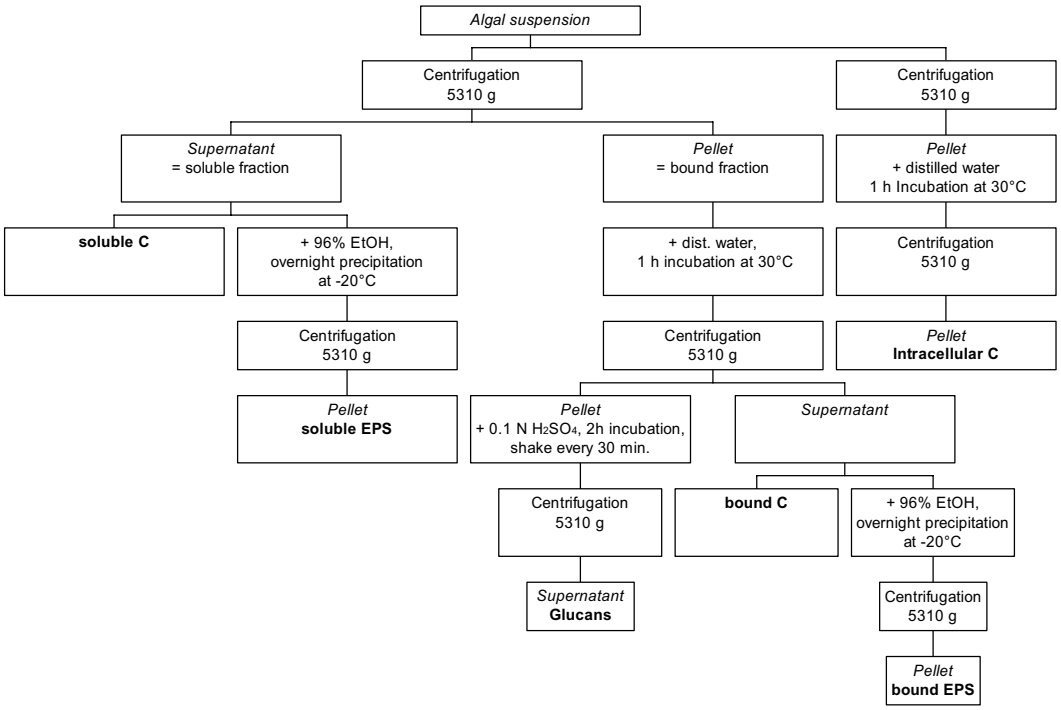

Figure 4.1. Flow chart of the fractionation of intra- and extracellular carbon and EPS.

Effect of bacteria

An experiment was carried out to study the effect of bacteria on partitioning of photosynthetically fixed carbon. In order to inhibit bacterial activity, an antibiotic cocktail (final concentration of 125 $\mu g \cdot ml^{-1}$ benzylpenicillin and 25 $\mu g \cdot ml^{-1}$ streptomycin sulfate) was added to one set microphytobenthos samples (n=2), 2 h before the experiment was started. Another set of microphytobenthos samples (n=1) was incubated without the addition of antibiotics. In order to check the inhibitory effect of the antibiotics, D-^{14}C-glucose (12.3 $kBq \cdot ml^{-1}$) was added to another set of samples. Using a photosynthetron (Lewis & Smith 1983) samples were incubated simultaneously at nine different irradiances (7, 23, 39, 84, 173, 322, 903, 1303 and 1903 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 15±1 °C. The samples were further processed as described above.

Statistical analyses

One-way ANOVA was used to test the effect of irradiance on the fractionation of photosynthetically fixed carbon. Statistical analyses were performed using the Statistica 5.1 software package.

Results

Comparison between the laboratory culture and the field sample

In order to compare the production of extracellular material between the field sample and the culture, values were normalized to chlorophyll *a*. The contents of chlorophyll *a* of the culture of *Cylindrotheca closterium* and in the field sample were 1.66 - 1.91 µg·ml^{-1} and 2.28-3.75 µg·ml^{-1}, respectively. Microscopic examination revealed that the culture of *C. closterium* was free of bacteria, whereas the field sample contained 5.3 x 10^5 bacteria·ml^{-1}.

Photosynthetic parameters showed higher values in the field sample of natural microphytobenthos compared to the culture of *C. closterium* (Table 4.1): P^B_{max} was about 2.5 times higher in the field sample, while the distinction in the other photosynthetic parameters was less pronounced.

There were no proportional differences in the amounts of intracellular and total extracellular carbon between the culture of *C. closterium* and the field sample of benthic diatoms. However, a large difference was observed in the amounts of the soluble and bound carbon fractions (Fig. 4.2, Table 4.2). The amount of soluble carbon was much higher in the field sample than in the culture while the opposite was true for the fraction of bound carbon. *C. closterium* and the field sample excreted on average 70 % and 75 % of total fixed carbon, respectively, even at low irradiances (≥ 5 µmol photons m^{-2} s^{-1}). The relative amounts of photosynthetically fixed carbon that were excreted did not vary to a large extend over the range of irradiances measured. It was constant for *C. closterium*, while in the microphytobenthos samples somewhat lower relative amounts were excreted at the highest irradiances ($F=3.58$; $p<0.05$).

Table 4.1. Photosynthesis parameters of *C. closterium* and natural microphytobenthos. P^B_{max} : maximum photosynthetic rate expressed per chlorophyll *a*; α^B: photosynthetic efficiency normalized to chlorophyll *a*; E_k: the light saturation parameter.

Parameters	*C. closterium*	Natural microphytobenthos
P^B_{max} [a]	1.15± 0.34	2.97± 0.56
α^B [b]	0.02± 0.01	0.04±0.003
E_k [c]	61.99± 22.19	74.17± 29.57

[a] mg C·mg Chl *a*$^{-1}$·h^{-1}

[b] mg C·mg·Chl *a*$^{-1}$·h^{-1}· (µmol photons m^{-2} s^{-1})$^{-1}$

[c] µmol photons·m^{-2}·s^{-1}

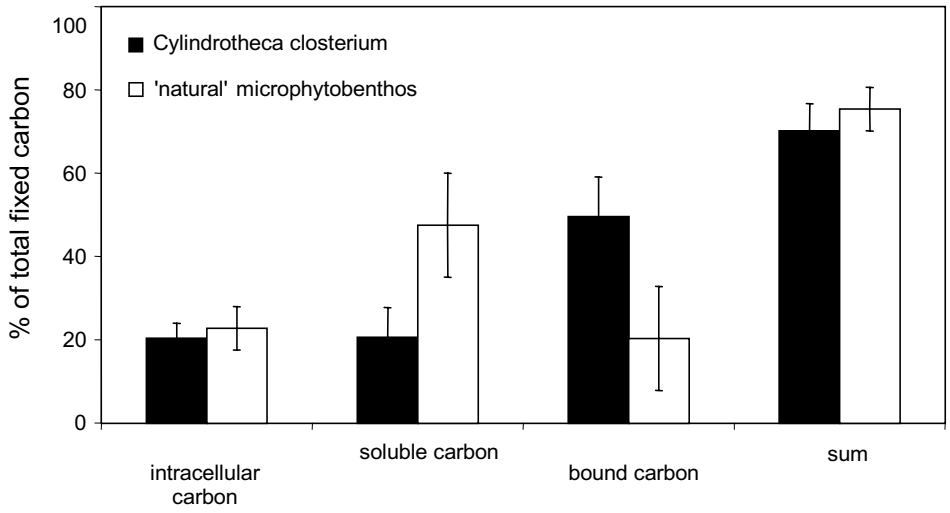

Figure 4.2. Amount of intra- and extracellular carbon (percentage of total fixed C) in a culture of *Cylindrotheca closterium* and in a field sample of benthic diatoms (Error bars indicate standard deviations, n=3).

In the field samples, the percentage of EPS (relative to extracellular carbon) was higher than in the culture; mean soluble EPS was 33±4 % of soluble carbon and mean bound EPS was 28±7 % of bound carbon. For *C. closterium* this was 21±7 % and 17±14 %, respectively. The percentages of soluble and bound EPS (relative to soluble carbon and bound carbon, respectively) were constant over the range of irradiances. This was true for both *C. closterium* and the natural microphytobenthos.

A constant percentage of 21 % of photosynthetically fixed carbon was recovered from the intracellular pool in the culture samples. This was on average 23 % in the field samples. However, in the field samples the percentage increased with increasing irradiance ($F=3.36$; $p<0.05$), (Table 4.2). The relative amounts of intracellular carbon were comparable between culture and the field samples (Fig. 4.2). In contrast, the average percentages of carbon recovered from the glucan fraction showed large differences between culture and field samples (9 % in *C. closterium* and 2 % in the field sample).

Light - response curves for the different carbon fractions are shown in Fig. 4.3 and Fig. 4.4 for *C. closterium* and natural microphytobenthos, respectively. In both samples, the total amount of fixed carbon increased with increasing irradiances, and the fits showed a saturation at approximately 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 4.3A, 4.4A). In the light saturated part of the curve, carbon fixation was constant for *C. closterium*, while it decreased for natural microphytobenthos. In both samples, the curves of the bound carbon fraction showed a similar

Table 4.2. Average relative carbon incorporation (relative to total carbon incorporation) of the analyzed intra- and extracellular carbon fractions in axenic cultures of *C. closterium* and natural microphytobenthos samples.

Mean irradiance [a]	Soluble carbon [b]	Soluble EPS [b]	Bound carbon [b]	Bound EPS [b]	Intra-cellular carbon [b]	Glucans [b]
C. closterium						
7±2	25.5	5.2	43.1	0.0	31.4	15.8
18±1	31.5	6.2	46.9	0.0	21.6	18.7
35±2	25.5	5.4	47.7	2.4	26.8	17.4
43±5	29.1	3.1	47.6	14.5	23.3	4.5
80±7	41.2	7.9	77.7	32.8	31.1	7.8
132±31	13.9	4.0	64.6	11.7	21.5	12.1
313±23	30.5	9.4	50.8	17.6	18.7	6.0
704±40	10.0	3.0	66.1	9.7	23.9	8.3
1196±84	14.4	1.9	68.6	24.0	16.9	9.0
Natural microphytobenthos						
7±2	47.0	13.3	33.5	10.0	19.5	2.1
18±1	66.4	25.2	11.9	6.0	21.7	1.9
35±2	69.4	26.6	10.6	2.9	20.0	1.4
43±5	52.2	25.4	29.5	7.7	18.4	2.4
80±7	63.9	22.9	18.5	4.8	17.6	1.7
132±31	36.9	11.1	41.6	7.2	21.5	2.1
313±23	61.2	29.0	7.6	2.8	31.2	2.1
704±40	38.7	12.5	33.3	10.4	28.0	1.7
1196±84	36.6	10.5	32.8	7.2	30.6	1.9

[a] µmol photons m^{-2} s^{-1}
[b] %

pattern compared to total carbon. This was also the case for the intracellular carbon fractions. The soluble carbon fraction increased up to an irradiance of 300 µmol·m^{-2}·s^{-1} and showed a concomitant decrease at higher irradiances in both samples. The content of glucans in the culture of *C. closterium* and the natural microphytobenthos samples saturated at an irradiance of 100 and 150 µmol·m^{-2}·s^{-1}, respectively, and remained constant in the saturating part of the curve. In Fig. 4.3B and 4.4B the values and fitted curves for bound carbon and EPS and soluble carbon and EPS are presented. The curves of soluble EPS showed the same pattern as soluble carbon for both samples, values increased until 300 µmol·m^{-2}·s^{-1} and then decreased.

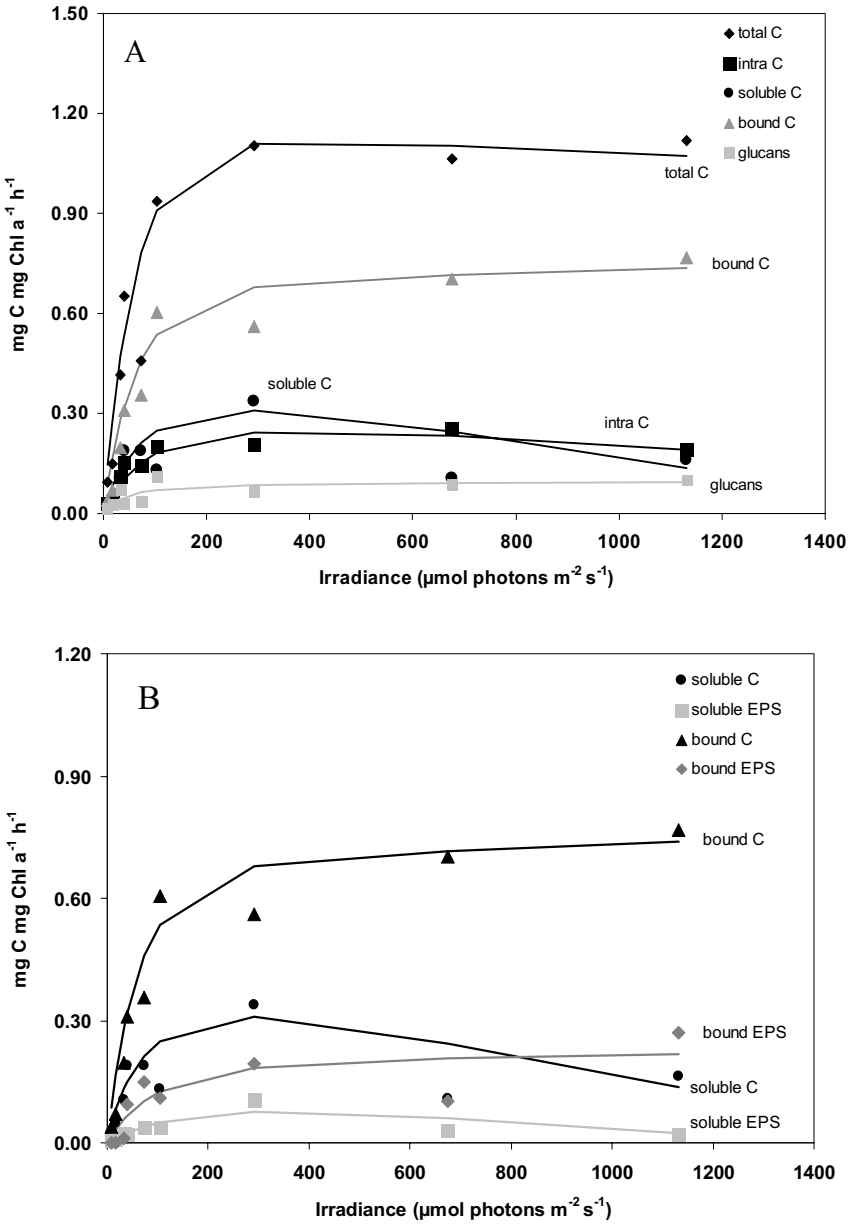

Figure 4.3. Total carbon fixation and partitioning in fractions of intracellular carbon, glucans, extracellular soluble and bound carbon (A), and in fractions of extracellular soluble and bound carbon and EPS (B) at different irradiances by *Cylindrotheca closterium*.

Considerable variation was observed in the values of bound EPS (see also Table 4.2), however, the curves showed comparable patterns between culture and natural microphytobenthos samples.

The α^B values obtained from the fits of the light – response curves give an indication of the efficiency of carbon incorporation in the different carbon fractions at limiting light intensities. The values of α^B differed between the culture and the natural microphytobenthos

samples for the total, bound and soluble carbon fractions and for the soluble EPS fraction (Table 4.3)

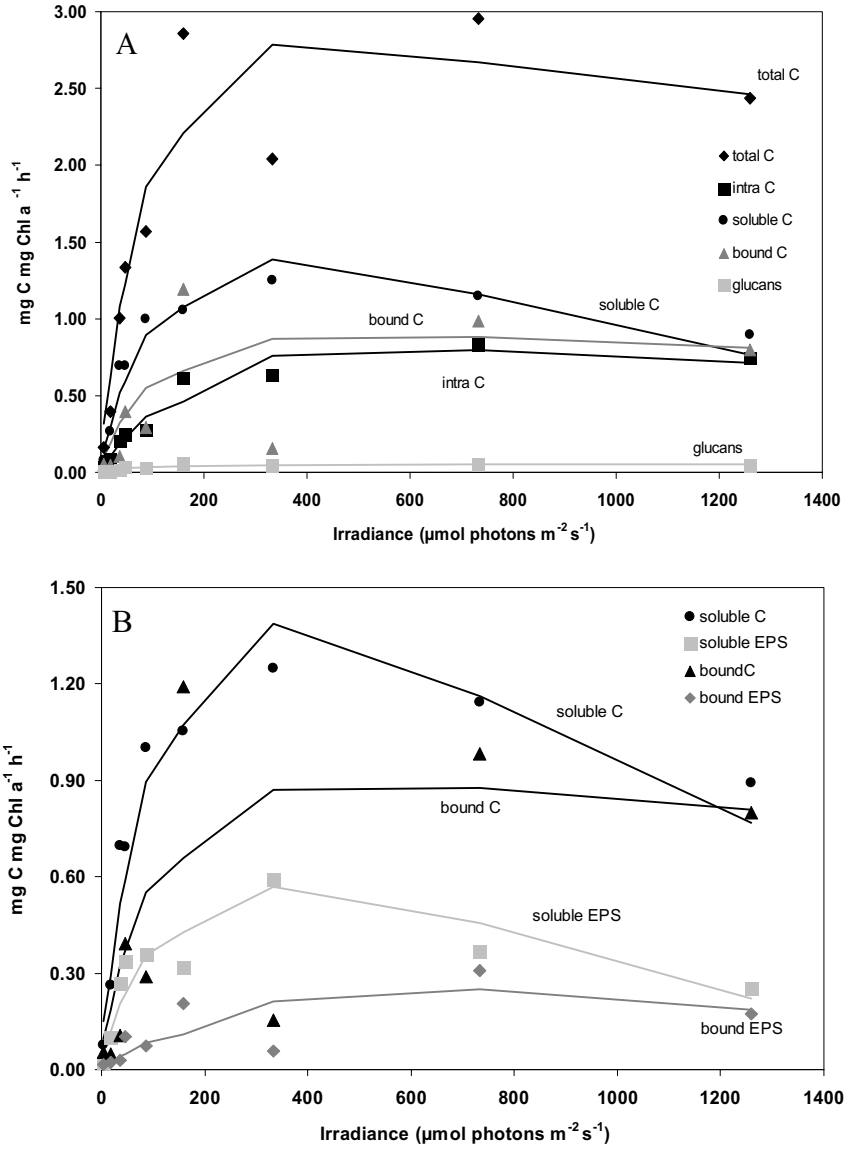

Figure 4.4. Total carbon fixation and partitioning in fractions of intracellular carbon, glucans, extracellular soluble and bound carbon (A), and in fractions of extracellular soluble and bound carbon and EPS (B) at different irradiances by a field sample of microphytobenthos.

Bacterial influence

The microphytobenthos sample contained 0.96 $\mu g \cdot ml^{-1}$ chlorophyll *a,* and 9.8 x 10^5 bacteria$\cdot ml^{-1}$ were counted. Maximum carbon fixation rates were considerably higher in the samples with antibiotics compared to the samples without antibiotics (P^B_{max}: 1.90 and 1.14 mg C·mg Chl $a^{-1} \cdot h^{-1}$), respectively. However, α^B-values only differed slightly between both

Table 4.3. Chlorophyll *a*-normalized photosynthetic efficiency values (α^B) obtained from the light – response curves for carbon fixation and carbon incorporation in intra- and extracellular carbon fractions at different irradiances for *C. closterium* and natural microphyobenthos (± standerd error, n=3).

	C. closterium	Natural microphytobenthos
	mg C·mg Chl $a^{-1} \cdot h^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$	
Total carbon	0.019±0.010	0.044±0.009
Intracellular carbon	0.004±0.003	0.006±0.001
Soluble fraction	0.004±0.001	0.020±0.011
Soluble EPS	0.001±0.0003	0.008±0.004
Bound carbon	0.005±0.003	0.012±0.005
Bound EPS	0.002±0.001	0.001±0.001
Glucans	0.002±0.002	0.001±0.0004

samples (0.006 and 0.008 mg C·mg Chl $a^{-1} \cdot h^{-1}$ $(\mu mol \cdot m^{-2} \cdot s^{-1})^{-1}$), (Fig. 4.4). The light saturation parameter E_k was as twice as high in the sample with antibiotics compared to the sample without antibiotics (316 and 142 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

The addition of antibiotics resulted in higher carbon incorporation in almost all carbon fractions (Fig. 4.5). The fitted curves of the different carbon fractions showed comparable patterns between samples with and without antibiotics, with the exception of intracellular carbon and the bound EPS fractions. Mean percentages of EPS in the soluble fractions were in the same range as those of the field sample in the previously conducted experiment, (25±14 % and 36±7 % for soluble EPS, and 9±3 % and 11± 9% bound EPS in the incubations with without antibiotics, respectively).

The efficiency of carbon fixation and EPS production (α^B) did not show large differences between the samples with and without the addition of antibiotics (Table 4.4). The

Table 4.4. Chlorophyll *a*-normalized photosynthetic efficiency values (α^B) obtained from the light – response curves for carbon fixation and carbon incorporation in intra- and extracellular carbon fractions at different irradiances in natural microphyobenthos with (n=2) and without (n=1) the addition of antibiotics.

	With antibiotics	Without antibiotics
	mg C·mg Chl a^{-1}·h^{-1}·(µmol photons·m^{-2}·s^{-1}) $^{-1}$	
Total carbon	0.006±0.001	0.008
Intracellular carbon	0.001±0.0001	0.001
Soluble carbon	0.004±0.0008	0.002
Soluble EPS	0.001±0.0003	0.001
Bound carbon	0.001±0.0005	0.001
Bound EPS	0.0001±0.00001	0.0001

α^B- values were comparable to those found in the previously conducted experiment, with the exception of the total fixed carbon and bound EPS fractions, which were considerably lower.

Discussion

Methodology

The relative amount of carbon incorporated in the total extracelular carbon fraction (sum of soluble and bound carbon) was similar to values reported by Goto et al. (1999). Smith & Underwood (2000) also found that up to 70% of the assimilated ^{14}C was present in the extracellular fraction. However, Smith & Underwood (2000) only analyzed the soluble carbon fraction. Therefore, their value is high when compared to the values reported by Goto et al. (1999) and to our results. Goto et al. (1999) only found about 3% of the fixed carbon in the soluble carbon fraction of different diatom species and in a mixed microphytobenthos sample. In our hands, the amounts of soluble and bound carbon varied between *C. closterium* and the field sample as they did with irradiance. The discrepancy between our results and those of Goto et al. (1999) may be explained by the fact that these authors used GF/F filters to

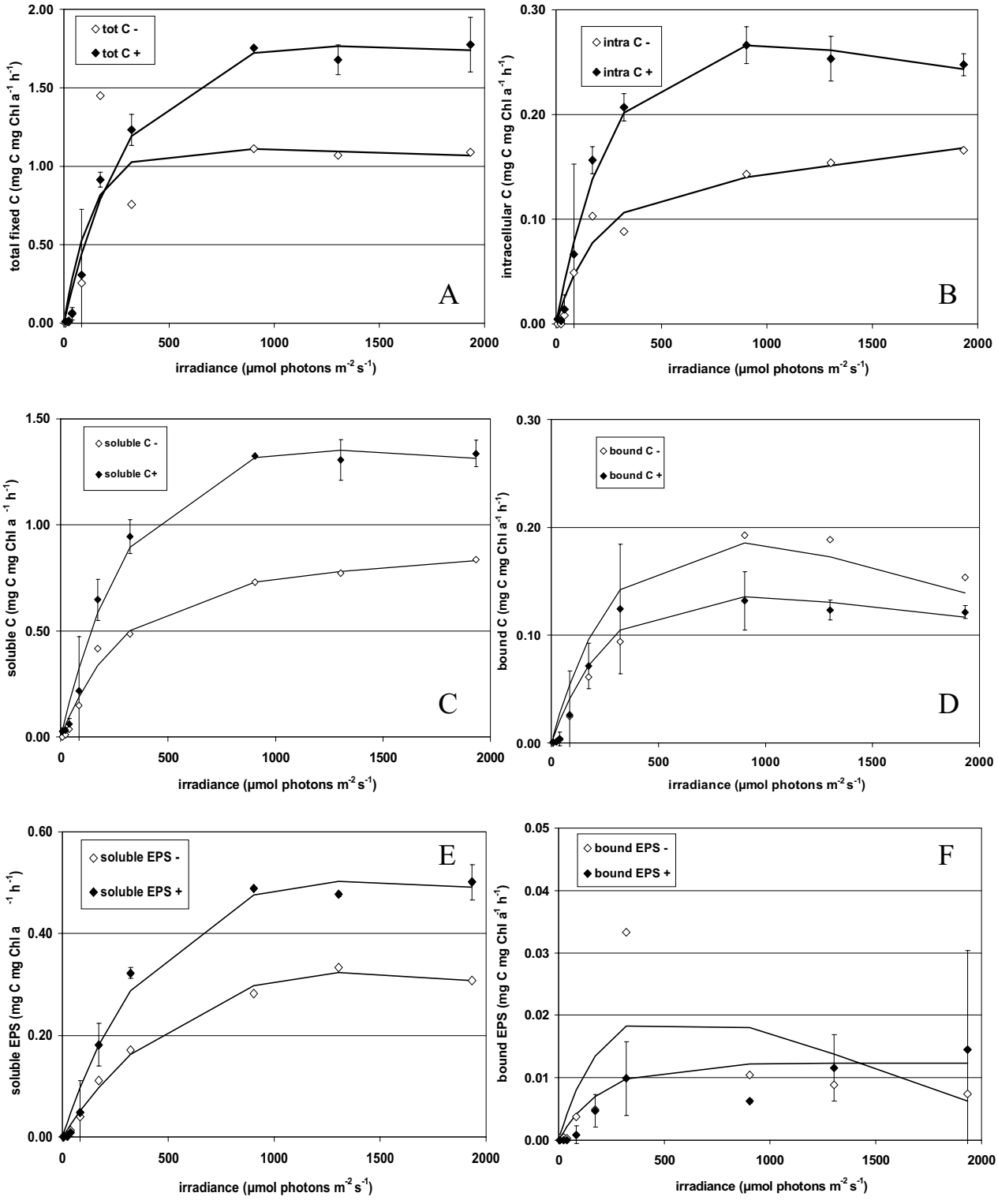

Figure 4.5. Carbon fixation and partitioning in total carbohydrate (A), intracellular carbohydrate (B), soluble carbohydrate (C), bound carbohydrate (D), soluble EPS (E) and bound EPS (F), at different irradiances by a field sample of microphytobenthos with (+) and without (-) addition of antibiotics (Error bars indicate standard deviations, n=2).

separate algae and supernatant, whereas we used centrifugation. Glass fiber filters are known to adsorb dissolved organic matter (Maske & Garcia-Mendoza 1994) and therefore the values reported by Goto et al. (1999) may be underestimated.

Goto et al. (1999) found higher ^{14}C activity in the intracellular carbon fraction (26-57 %) when compared to our study. One explanation might be that the amount of carbon that is stored within the cells is species specific (Goto et al. 1999; Staats et al. 1999).

Comparison between an axenic culture and a field sample

In order to examine the effect of single environmental factors on excretion processes in benthic diatoms, experiments with axenic cultures are usefull. However, when natural communities of microphytobenthos are studied, bacteria are naturally present. Therefore, a comparison between results obtained by an axenic laboratory culture and by a sample collected from the field is difficult. In literature, examples of such comparisons have been described (Smith & Underwood 1998; Staats et al. 2000a). However, in these studies the laboratory culture and the field sample were treated differently. In our study the field samples were kept under exactly the same conditions as the culture one day before the experiment started and during the experiment. Nevertheless, the history of the cells was different. *C. closterium* was grown on sand and in liquid medium under constant light and temperature conditions, while the natural community experienced varying field conditions. Notwithstanding the different histories, the total amount of irradiance did not differ much between the culture and the field sample during the 10 days preceding the experiment. The culture received a total irradiance of 4.3 $mol \cdot m^{-2}$ during pre-incubation, whereas this was 5.0 $mol \cdot m^{-2}$ for the field sample (height of the station and tidal effects were included in the calculations) before it was taken. Although the total dose of irradiance was more or less similar for the two samples, they experienced large differences in the duration of light or darkness as well as in the maximum irradiance.

The second important difference was the presence or absence of bacteria in the algal suspensions. The culture of *C. closterium* was bacteria free, whereas the field sample contained about 5×10^5 $bacteria \cdot ml^{-1}$. Bacteria may affect the amount of extracellular carbon in the samples because they both produce and decompose extracellular products (Cole et al. 1988; Decho 1990). In order to assess the influence of bacteria, an experiment was conducted in which antibiotics were added which successfully inhibited bacterial activity. This was demonstrated by the absence of ^{14}C incorporation from added ^{14}C-glucose. Therefore, results of the field sample treated with antibiotics can be considered as true algal excretion. The observation that there was no obvious difference in the efficiency of carbon fixation (α^B) between the samples with and without addition of antibiotics, was taken as evidence that the

algae were not affected by the addition of antibiotics. In the samples treated with antibiotics, we observed higher carbon incorporation in the total, intracellular and soluble carbon fractions as well as in the soluble EPS fraction. This was not the case in the bound carbon and bound EPS fractions. So far, we do not have an explanation for the observed results.

The relative amount of photosynthetically fixed carbon that was excreted was similar between the culture and the field samples. However, the contribution of bound and soluble carbon to the total extracellular carbon pool differed. A reason for this may be that the separation between the soluble and bound carbon fractions is operational. In reality the transition between these two fractions could be less strict. Furthermore, the production of these types of extracellular carbon depends on the physiological status of the organism (chapter 2, 3), which may explain the distinction between the field and the culture samples. In the field sample slightly more carbon was incorporated in the EPS fractions compared to the culture. The amounts of EPS that are excreted may differ between algal species as a consequence of differences in photosynthetic and EPS production capacities (Smith & Underwood 1998; Goto et al. 1999). Secondly, the algae from the field were not in a defined growth stage as was the case for *C. closterium.* The amounts of produced EPS are obviously dependent on the physiological state of the organism and the growth phase of the culture (Staats et al. 1999, chapter 2, 3).

The differences in the initial slope of the light - response curves (α^B) between the culture and the field sample are difficult to interpret. However, the higher capacity of the field samples to fix carbon and to produce exopolymers, may be the result of differences in species composition, growth status or light history. Notwithstanding these differences the percentages of soluble and bound EPS were constant over the range of irradiances measured. This means the production of EPS is directly dependent on the rate of photosynthesis.

Conclusions

Although some differences between a laboratory culture and a sample from the field were observed, the relative amount of total excreted carbon (measured as the sum of soluble and bound carbon) as well as the percentage of EPS in these fractions were comparable between an axenic culture of *C. closterium* and a field sample. Photosynthetically fixed carbon was excreted within a period of 30 min, even under low light conditions (less than 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The percentage of excreted carbon relative to total fixed carbon was constant for *C. closterium*, while it changed only slightly at higher irradiances in the natural microphytobenthos. This suggests that a mechanism of photosynthetic overflow mechanism as a result of nutrient limitation (Staats et al. 1999) did not apply in these experiments. The production of extracellular material was directly dependent on photosynthetic rates, which corroborates the findings of Smith & Underwood (2000) and Staats et al. (2000a). Staats et al.

(2000a) observed that a certain light limit was required for the production of EPS. In contrast, a number of studies reported production of exopolymers in the dark (Smith and Underwood 2000; chapter 2, 3). In our study no measurements were undertaken in the dark, however, the amount of light that was provided affected the quantity of carbon that was excreted.

The results obtained in this study indicate a direct relationship between irradiance and the amount of carbon that was excreted. This means that in the field there might be a seasonal effect of irradiance on the production of exopolymers, which is closely related to the variations in the rate of photosynthesis.

CHAPTER 5

SHORT-TERM DYNAMICS IN MICROPHYTOBENTHOS DISTRIBUTION AND ASSOCIATED EXTRACELLULAR CARBOHYDRATES IN SURFACE SEDIMENTS OF AN INTERTIDAL MUDFLAT

J.F.C. de Brouwer & L.J. Stal

Abstract

Two field studies were conducted to study the *in situ* net production of extracellular carbohydrates and the distribution of benthic diatoms over 24 h periods. A comparison was made between a situation where a clear surface biofilm of diatoms had developed and a situation where this was not the case. Vertical profiles were made by sampling the top 2 mm of the sediment at depth intervals of 0.2 mm using the cryolander technique. In the presence of a biofilm, diatom distribution showed a consistent pattern when the sediment was emersed. In the light, most of the diatoms were present in the top 0.2 mm while in the dark diatoms were homogeneously distributed in the upper 2 mm of the sediment. When a biofilm was absent, no clear patterns were observed. Extracellular carbohydrates were extracted from the sediment and separated in two operationally defined fractions (water- and EDTA-extractable). The two carbohydrate fractions showed a different dynamic behavior. The water-extractable carbohydrate fraction was highly variable while the EDTA-extractable fraction behaved more conservatively. Only in the light and in the presence of a diatom biofilm, production of extracellular carbohydrates was observed. The maximum rate of chlorophyll-normalized production of extracellular carbohydrates, expressed in glucose equivalents ($g \cdot g^{-1}$), amounted to 20 h^{-1} in the upper 0.2 mm. The molecular size distribution of both carbohydrate fractions was similar. The monosaccharide composition was also similar, except that the EDTA-extractable fraction contained a higher percentage of uronic acids. Carbohydrates produced during tidal emersion were rich in glucose and were rapidly turned over.

Introduction

Benthic epipelic diatoms are the most important group of primary producers in intertidal mudflats (Admiraal 1984; Smith & Underwood 1998). They are known to produce copious amounts of Extracellular Polymeric Substances (EPS) mainly consisting of carbohydrates (Hoagland et al. 1993). By doing so, diatoms form a biofilm that serves to produce their own micro-environment that protects them from the rapidly changing conditions in intertidal mudflats (Decho 1994). The excretion of EPS plays a role in the movement of epipelic benthic diatoms (Edgar & Pickett-Heaps 1984) and it allows the organisms to adhere to sediment surfaces (Wang et al. 1997). EPS-production may also be controlled by nutrient availability, for instance when the organisms grow under unbalanced conditions (Ruddy et al. 1998a, b), a situation likely to occur in intertidal environments (Flothmann & Werner 1992). The presence of diatom biofilms increases the stability of the sediment surface (Paterson 1989; Kornman & de Deckere 1998) and can have a profound effect on the morphodynamics of mudflats (Dyer 1998; chapter 6). Through the excretion of EPS, diatoms are responsible for a considerable input of high quality organic carbon into the sediment that may be utilized as a food source for heterotrophic consumers (King 1986; van Duyl et al. 1999).

The dynamics of extracellular carbohydrates in intertidal mudflats have been studied at different temporal and spatial scales (Underwood & Paterson 1993a; Taylor & Paterson 1998; Taylor et al. 1999; Staats et al. 2000a; chapter 6, 7). By using different extraction protocols, different operationally defined carbohydrate fractions are obtained (Underwood et al. 1995). Water-extractable (also called colloidal) carbohydrate, a water-soluble fraction that is commonly used for the determination of extracellular carbohydrates, shows a significant correlation to chlorophyll *a* in intertidal mudflats that are dominated by epipelic diatom assemblages (Underwood & Smith 1998b; chapter 7). Other fractions were obtained using EDTA to recover extracellular carbohydrates that are more tightly associated with cell walls or sediment particles (Decho & Lopez 1993; de Winder et al. 1999). Finally, total hydrolyzable carbohydrate contents can be measured by direct hydrolysis of sediment samples (Taylor & Paterson 1998; Taylor et al. 1999). Production of extracellular carbohydrates has been observed to occur when the sediment was exposed in the light (de Winder et al. 1999; Taylor et al. 1999, Staats et al. 2000a) and is dependent on a number of factors including time of exposure (Underwood & Paterson 1993a), growth stage (Staats et al. 1999; Smith & Underwood 2000) and morphology of the mudflat (Taylor & Paterson 1998; chapter 7). Furthermore, increased production of extracellular carbohydrates was observed at moments when diatom numbers at the mudflat surface changed, typically at the start and end of the emersion period (Smith & Underwood 1998, Underwood and Smith 1998a).

To elucidate the factors that control the excretion of carbohydrate, it is important to sample in a way that provides sufficient resolution within the area were diatoms are active (Paterson et al. 2000). Epipelic diatoms show rhythmic migrations in response to light and

tidal cycles (Happey-Wood & Jones 1988; Serôdio et al. 1997) and the amplitude of vertical migration has been reported to vary between 1.6 mm (Paterson 1986) and 12 cm (Kingston 1999). In intertidal mudflats, migration generally occurs within the upper 2 mm of the sediment. The cryolander method (Wiltshire et al. 1997) is a sampling technique that allows the cross sectioning of undisturbed sediment cores at a depth resolution of up to 100 μm and is therefore the method of choice to investigate carbohydrate and diatom dynamics in surface sediments.

In situ studies conducted have so far measured only daytime emersion periods (Taylor et al. 1999) or lacked sufficient vertical resolution when comparing day- and nighttime emersion periods (van Duyl et al 1999; Staats et al 2000a). In addition, there is hardly any information about the biochemical properties of extracellular carbohydrates in mudflat sediments. Information about the monosaccharide composition of carbohydrates excreted by benthic diatoms comes mainly from work on cultures (Staats et al. 1999; de Winder et al. 1999), although Taylor et al. (1999) analyzed the monosaccharide composition of water-extractable and bulk carbohydrates in the Eden estuary in Scotland.

In addition, molecular size distribution analyses can provide information about the properties of extracellular carbohydrates and may therefore be useful to understand the dynamics of these sugars in intertidal mudflats. Ethanol precipitation of water-extractable carbohydrates is often used in order to obtain the high molecular weight fraction which is termed EPS (Underwood et al. 1995) and is composed of the >100 kD polysaccharides (own unpublished observations). However, in marine sediments it has been demonstrated that polysaccharides are rapidly decomposed, resulting in smaller polymers with a broad range in molecular weights (Arnosti 1995; Arnosti 1996) that are well below the size that precipitates in ethanol. Furthermore, EPS-excreting organisms may produce polymers that are variable in chain length. For example, Bender et al. (1994) found that the benthic mat forming cyanobacterium *Oscillatoria* sp. excreted polysaccharides of two different sizes.

In this paper, diurnal dynamics in diatom distribution and extracellular carbohydrate in the upper 2 mm of intertidal mudflat sediments are described. The main questions that we address are: 1) what are the effects of light and tidal inundation on diatom distribution and carbohydrate standing stocks? 2) What are the dynamics of extracellular carbohydrates in terms of biochemical properties? 3) Are the two extracellular carbohydrate fractions comparable in terms of dynamics and biochemical properties?

The studies that were conducted represented two different situations that may occur in intertidal mudflats. In June 1998 the mudflat was devoid of a distinct visual layer of microphytobenthos, while in May 1999 extensive mats were observed that altered the sediment properties of the mudflat surface, changing bedform structure and increasing sediment stability (chapter 6).

Material and methods

Site description

The Biezelingse Ham mudflat is situated along the northern shore of the Westerschelde estuary in the South-West of the Netherlands (51°56.47'N, 3°55.51'E). The mudflat has an area of about 1.5 km^2 and the tidal range is about 4 m. A small saltmarsh and a freshwater inlet are situated at the north side of the mudflat. Sampling was carried out at the middle station of a transect established at the southern end of the mudflat. The emersion time at this site was approximately 6 h. Sediment at this part of the mudflat was typically muddy with a median grainsize < 20 μm and the surface was planar with major bedforms generally absent. A detailed survey, examining the dynamics in biology and sedimentology of this mudflat was carried out in chapter 6.

Sampling methodology

The sampling site was sampled twice for a period of 24 h. In both studies low tide during daytime emersion occurred at 14:00 h, while low tide during nighttime emersion occurred at 2:30 h. The first study took place on 16 June 1998, about 3 weeks after the collapse of a benthic diatom bloom (see also chapter 6). At that time, no diatom biofilms were found at the sediment surface. In contrast, well-developed diatom films were present during the second study carried out on 20 May 1999. Pigment analysis in the sediment samples revealed that in both studies diatoms were by far the dominant phototrophic organisms present. In May 1999, sediment samples were taken every 30 or 60 min, starting 15 minutes after the sediment became exposed. The last sample was taken 15 minutes before immersion. In June 1998, 3 sediment samples were taken during daytime emersion (1:25, 4:00, 5:00 h after emersion), while 7 cores were taken during nighttime emersion (15 min – 5.5 h after emersion at regular time intervals).

Sediment cores were collected using the cryolander coring method (Wiltshire et al. 1997). Briefly, the sediment surface was frozen with the vapor of liquid N_2 resulting in a minimal disturbance of the structure of the sediment. After the sediment surface was frozen, liquid N_2 was poured in order to the freeze deeper sediment layers (up to 1 cm). The frozen sediment samples could easily be removed and were wrapped in aluminium foil and transported to the laboratory in liquid N_2. Prior to sectioning, the cryolander samples were cut into pieces of approximately 1 cm^2 by using a diamond grinder. Subsequently, the top 1 mm of the sediment was sectioned in 0.2 mm slices and the second mm was sectioned in 2 slices of 0.5 mm using a freeze-microtome (Leica, SM 2000 R). Samples were lyophilized prior to the analysis of carbohydrate (n=3) and chlorophyll *a* (n=3).

Results are expressed as contents ($\mu g \cdot g^{-1}$ dry weight of sediment) and would be sensitive to changes in water content. In the two field studies conducted, water content (calculated from the wet and dry weight of the sediment) decreased with depth (from 55.5±8.4 and 75.1±6.5 % in the 0-0.2 mm depth horizon to 47.6±5.0 and 70.0±4.7 % in the 1.5-2.0 depth horizon, respectively in June 1998 and May 1999). Importantly, over the course of the two field studies water contents in the separate depth layers did not change, and therefore the time series were not influenced by the water content.

Chlorophyll a-analysis

Lyophilized sediment samples (10-100 mg) were extracted with 2 ml 90 : 10 (v/v) acetone : water for 1 h at 0°C. Extraction was aided by sonication. The extracts were centrifuged for 10 min at 1000 g. Chlorophyll *a* was measured fluorometrically using the equations of Lorenzen, applying a correction for pheopigments (Lorenzen 1966).

Carbohydrate analysis

The lyophilized sediment (5-55 mg) was extracted with 400 µl distilled water for 1 h at 30°C. The extract was centrifuged for 5 min at 6000 g and the water-extractable carbohydrate fraction was determined in the supernatant. Subsequently, the pellet was incubated with 500 µl 0.1 M Na_2EDTA for 16 h at room temperature. The extract was centrifuged for 5 min at 6000 g and the EDTA-extractable carbohydrate fraction was assayed in the supernatant. Carbohydrate was measured spectrophotometrically using the phenol-sulfuric acid assay (Dubois et al. 1956). Glucose was used as reference. To 200 µl sample, 200 µl 5% (w/v) phenol and 1 ml of concentrated sulfuric acid were added and the mixture was incubated for 35 min.

Size distribution and monomeric composition of carbohydrate fractions

Sediment samples taken 15 min after the start and 15 min before the end of the daytime emersion period of the May 1999 study were used for molecular size distribution analyses and determination of the monomeric carbohydrate composition. Samples were taken in the depth intervals of 0-0.2, 0.6-0.8 and 1.5-2.0 mm. Ten replicate samples were pooled, lyophilized and subsequently extracted with 1 ml distilled water and 1.5 ml 0.1 M Na_2EDTA as described above.

The size distribution was determined by ultrafiltration using Centricon centrifuge tubes (Millipore) with molecular weight cut-off filters of 100, 50, and 10 kD. These filters are commonly calibrated using protein standards. Carbohydrates have different space filling

molecular properties and therefore molecular weight cut-off may be slightly different. Consequently, the size classes must be regarded as operationally defined. Samples were centrifuged for 30 min, 1000 g; 15 min, 5000 g; and 2 h, 5000 g, through 100, 50 and 10 kD filters, respectively. The fractions retained by these filters were desalted adding 2 ml of distilled water and repeating the ultrafiltration. The recovery was determined by weighing the amount of original sample in the ultrafiltration device, the amount of filtrate and retentate after ultrafiltration and the carbohydrate concentrations in these fractions. Recoveries of the ultra filtration varied between 85 and 115% and were normalized to 100%.

The monosaccharide composition of the carbohydrates was determined by HPLC. Methanolysis and subsequent hydrolysis of the carbohydrates was carried out according to de Ruiter et al. (1992). Briefly, lyophilized samples were methanolyzed with 0.5 ml 2 M HCl in methanol for 16 h at 80°C. Subsequently, the methylglycosides were hydrolyzed to the monosaccharides with 2 M trifluoro-acetic acid (1 h at 120°C). The sugars where separated on a Carbopack-1 column (Dionex) and detected by a Pulse Amperometric Detector (PAD). Neutral sugars were separated running the following gradient: 0-20 min, 15 mM NaOH; 20-25 min, 300 mM NaOH, 25-35 min, 15 mM NaOH. Uronic acids were analyzed in a separate run using 100 mM NaOH/ 100 mM sodium acetate as eluent. The peaks of xylose and mannose co-eluted. Quantification was based on the assumption that both monosaccharides were present in equal amounts. The analytical reproducibility was within ±5% (n=8) for all monosaccharides analyzed. The relative contribution of the different monosaccharides was calculated using external standards and is expressed in weight%.

Statistical analyses

Statistical analysis was performed on log(n+1) transformed data. A MANOVA (multiple analysis of variance) design was used to test the effects of duration of emersion and day or night on the content of carbohydrates and chlorophyll *a*. Dependent variables were the content of chlorophyll *a* and carbohydrate at all depth intervals. The duration of emersion, day/night situation and the interaction were used as independent variables, where time is a numerical and day/night a categorical variable. The interaction between the duration of emersion and the day/night situation examines to what extent the evolution of the measured contents over the daytime emersion period are different from that measured during the nighttime emersion period. F-tests for the total depth layer were based on Wilkinsons Lambda F-statistics. Statistical results are given for the total depth layer, unless stated otherwise. The data were analysed using the Systat 5.02 package.

Results

Extracellular carbohydrates

In June 1998, the dry sediment content of water-extractable carbohydrate over the whole depth layer analysed was in average 206 ± 32 $\mu g \cdot g^{-1}$ while the EDTA-extractable carbohydrate amounted 670 ± 201 $\mu g \cdot g^{-1}$ (Fig. 5.1). There was no interaction between duration of emersion and day/night in either of these carbohydrate fractions (water-extractable carbohydrates, $F_{(7, 11)}=0.49$, $p=0.83$; EDTA-extractable carbohydrates, $F_{(7, 10)}=2.02$, $p=0.15$).

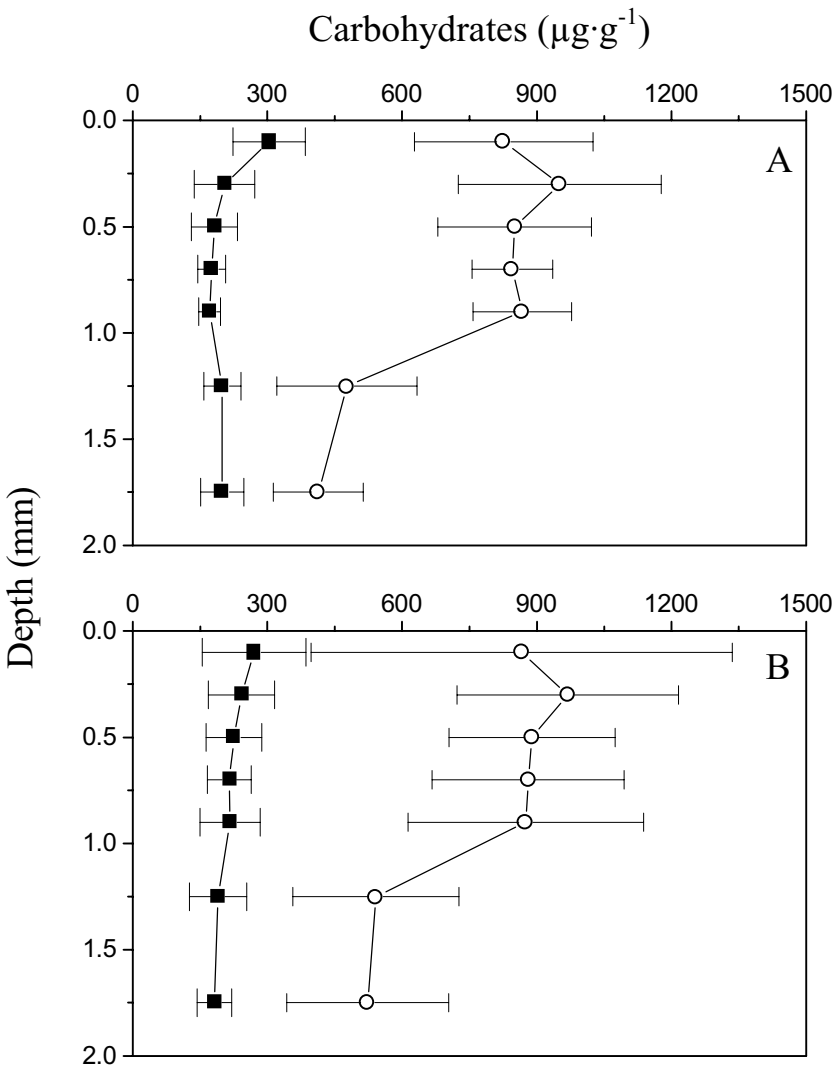

Figure 5.1. Vertical profiles (means ± 1 SD) of the average content of water- (■) and EDTA-extractable carbohydrates (○), 16 June 1998. (A); during the daytime emersion period, (B); during the nighttime emersion period.

The contents of both carbohydrate fractions did not vary over the emersion periods (water-extractable carbohydrates, $F_{(7, 11)}=0.49$, $p=0.83$; EDTA-extractable carbohydrates, $F_{(7, 10)}=2.31$, $p=0.11$). The content of water-extractable carbohydrate differed between the day- and nighttime emersion period ($F_{(7, 12)}=6.09$, $p<0.01$). However, when the separate depth intervals

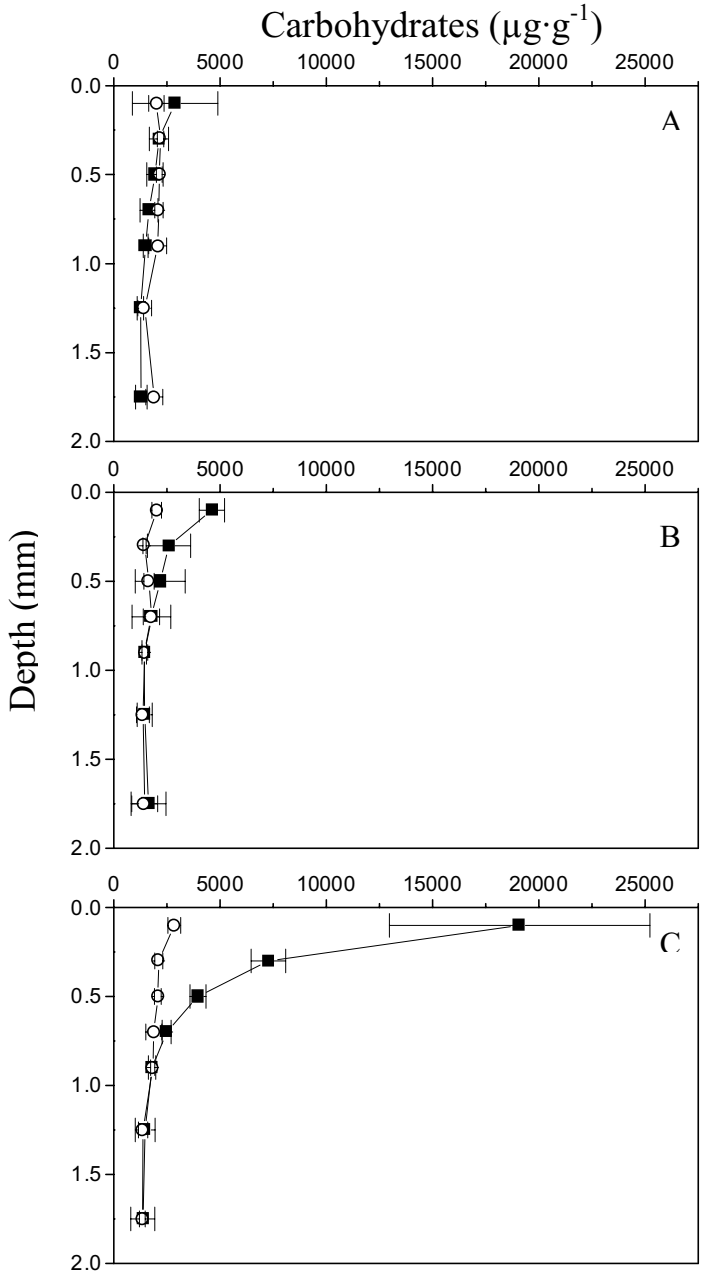

Figure 5.2. Vertical profiles (means ± 1 SD) of water- (■) and EDTA-extractable carbohydrates (○) over the daytime emersion period on 20 May 1999. (A); 15 min after emersion, (B); 2.05 h after emersion, (C); 5.5 h after emersion.

were considered, none of the tests revealed significant differences ($F_{(1, 18)}$<3.48, p>0.08 for all depth intervals). Vertical profiles of water-extractable carbohydrate showed no significant differences with depth. In contrast, the content of EDTA-extractable carbohydrate decreased from 900 µg·g^{-1} at the surface to 600 µg·g^{-1} below the depth of 1 mm (Fig. 5.1).

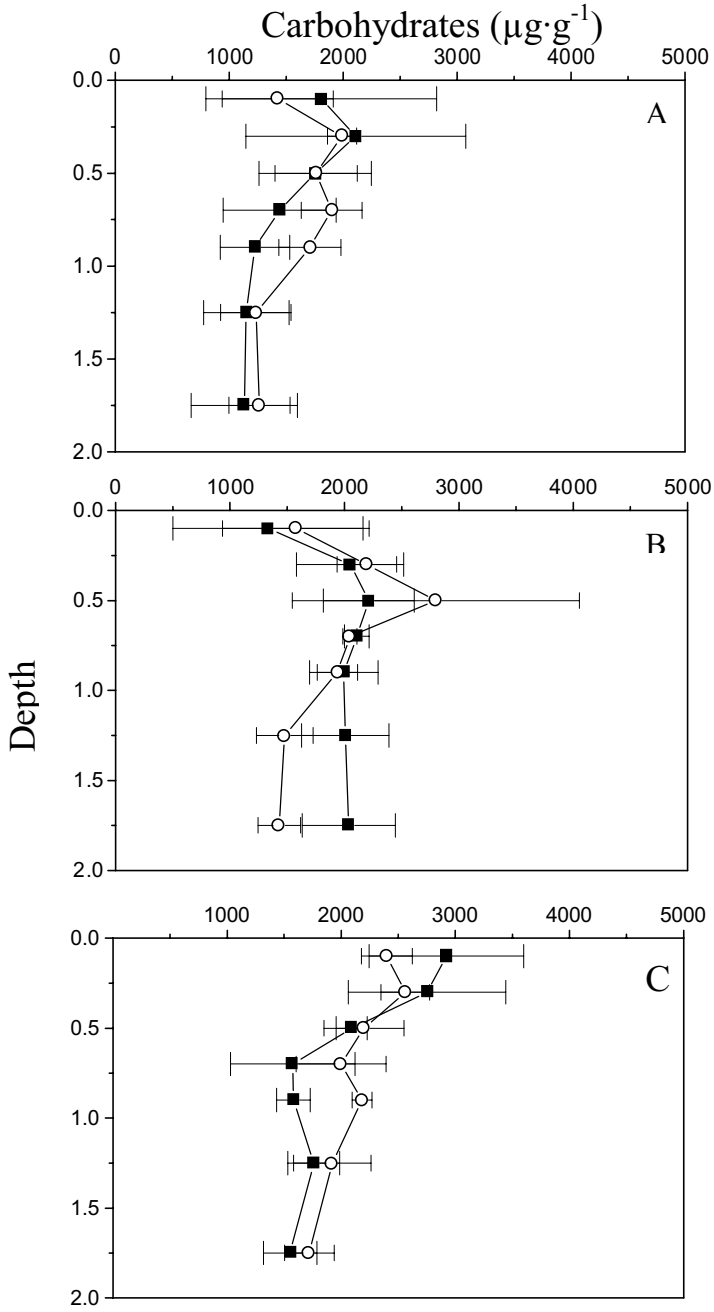

Figure 5.3. Vertical profiles (means ± 1 SD) of water- (■) and EDTA-extractable carbohydrates (○) over the nighttime emersion period on 20 May 1999. (A); 5 min after emersion, (B); 2.05 h after emersion, (C); 5.5 h after emersion.

In May 1999, the content of both water- and EDTA-extractable carbohydrate was higher than to the June 1998 situation. At the start of the emersion, water-extractable carbohydrate in the top 2 mm was on average 1644 ± 443 $\mu g \cdot g^{-1}$ while it was 1893 ± 305 $\mu g \cdot g^{-1}$ for EDTA-extractable carbohydrate. In the light, the content of water-extractable carbohydrate increased almost 10-fold in the upper 0.2 mm (Fig. 5.2). When considering the whole sampled depth layer, the interaction between duration of emersion and day/night was significant ($F_{(7, 36)}$=5.59, p<0.001). This was the result of the production of carbohydrates taking place during daytime emersion while this was not the case during nighttime emersion (see also Fig. 5.2 and 5.3). When the different depths were examined individually, a significant interaction effect was evident in the top 1 mm of the sediment ($F_{(1, 42)}$=5.6, p=0.022 at 0.8-1.0 mm). The interaction between time of emersion and day/night situation was not significant for EDTA-extractable carbohydrates ($F_{(7, 37)}$=0.67, p=0.70) and the amount did not vary during emersion ($F_{(7, 37)}$=1.75, 9=0.13). A significant increase in EDTA-extractable content during emersion was observed only in the top 0.2 mm ($F_{(1, 43)}$=7.35, p=0.01). The content of EDTA-extractable carbohydrate was higher over the daytime emersion compared to the nighttime emersion ($F_{(7, 37)}$=5.43, p<0.001), but again, this was mainly due to the higher

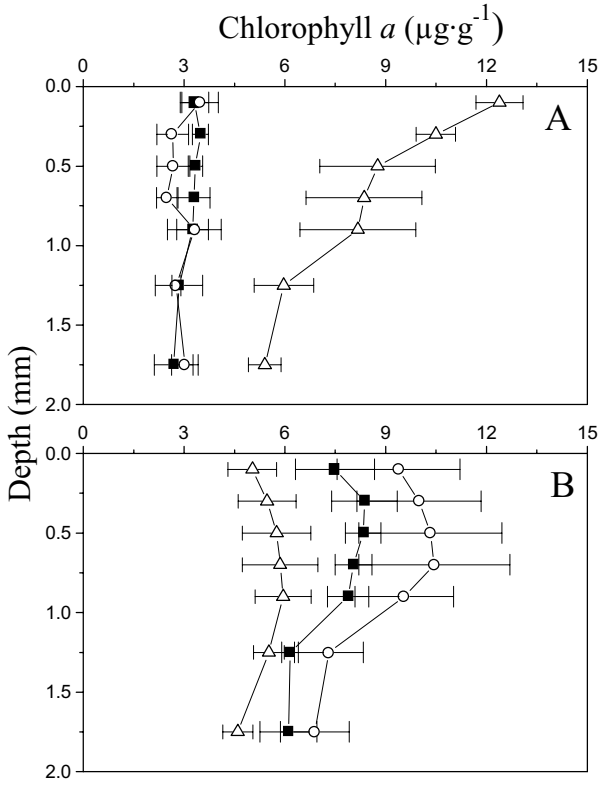

Figure 5.4. Vertical profiles (means ± 1 SD) of chlorophyll *a* contents, June 1998. (A); during the daytime emersion period, (B); during the nighttime emersion period, 1.25 h after emersion (■), 4 h after emersion (○) and 5 h after emersion (Δ).

content in the upper0.2 mm layer during the daytime emersion ($F_{(1, 43)}$=29.96, p<0.001). At the start of the nighttime emersion period, the carbohydrates that were produced in the light had disappeared and the levels had dropped to those at the start of the daytime emersion period (Fig. 5.2 and 5.3). During the night, the content of water- as well as EDTA-extractable carbohydrate did not vary with average values of 1648 ± 408 and 1610 ± 277 $\mu g \cdot g^{-1}$, respectively.

Chlorophyll a contents

In June 1998, the chlorophyll *a* content in the top 0.2 mm of the sediment during emersion varied between 3.3 and 12.3 $\mu g \cdot g^{-1}$ during daytime and 5.0 and 9.4 $\mu g \cdot g^{-1}$ during nighttime. The chlorophyll *a* content varied only marginally during the emersion periods ($F_{(7, 5)}$=4.86, p=0.05). When the daytime emersion was compared to the nighttime emersion, no significant changes were observed ($F_{(7, 5)}$=3.11, p=0.12), (Fig. 5.4).

In May 1999, the content of chlorophyll *a* was 5-10 fold higher when compared to the June 1998 situation. In the top 0.2 mm, the content of chlorophyll a during emersion was typically in the range of 155 $\mu g \cdot g^{-1}$ during the day and 51 $\mu g \cdot g^{-1}$ during the night. Although there were no significant changes between samples within the emersion periods ($F_{(7, 30)}$=0.41, p=0.89), vertical profiles differed markedly between day and night ($F_{(7, 30)}$=31.23, p<0.001), (Fig. 5.5). In the light, the content of chlorophyll *a* was highest in the top 0.2 mm decreasing

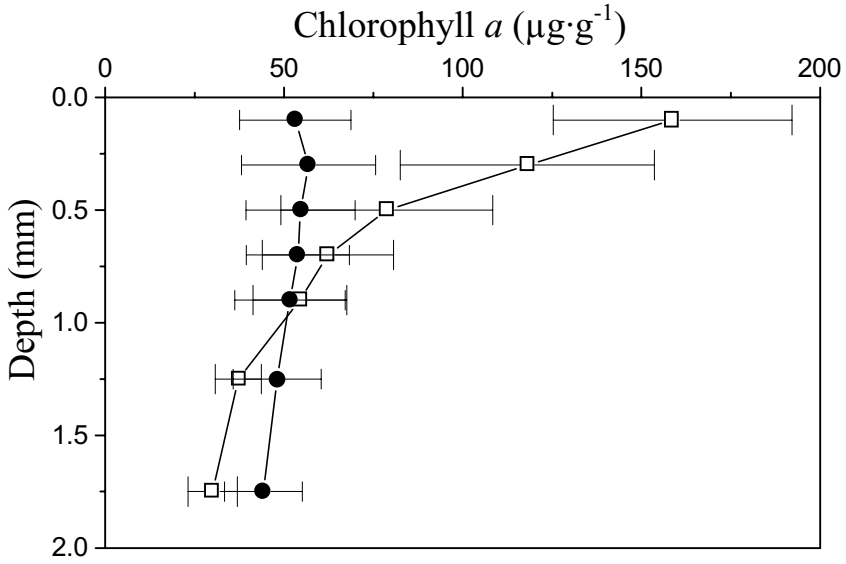

Figure 5.5. Vertical profiles (means ± 1 SD) of the average chlorophyll *a* contents on 20 May 1999 during the daytime emersion period (□) and the nighttime emersion period (●).

steeply with depth. During the night, the content of chlorophyll *a* remained constant over the depth range analyzed. The content of chlorophyll *a* in the top 2 mm during the night was about 75 % of the content during daytime.

Carbohydrate production in the light

During daytime emersion in May 1999, the carbohydrate content was normalized to chlorophyll *a* in order to obtain production estimates of extracellular carbohydrates at the measured depth intervals (Fig. 5.6, Table 5.1). Water-extractable carbohydrate production was significant in the top 1 mm, being highest in the top 0.2 mm. In this layer the chlorophyll-normalized production rate of water-extractable carbohydrate (in glucose equivalents) was 20 h^{-1}. For EDTA-extractable carbohydrates a significant increase was only found in the top 0.2 mm sediment layer. However, compared to water-extractable carbohydrates, production of EDTA-extractable carbohydrates was low (1.2 h^{-1}). Typically, the intercepts of the linear regressions for both water- and EDTA-extractable carbohydrates increased with depth (Table 5.1).

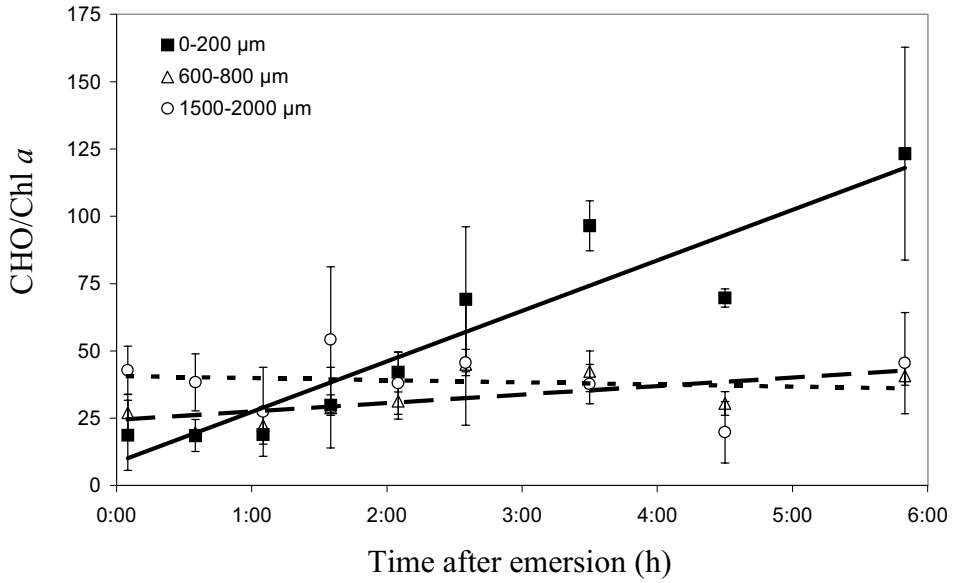

Figure 5.6. Average (± 1 SD) chlorophyll *a*-normalized water-extractable carbohydrate production at the three sampled depth intervals during the daytime emersion period on 20 May 1999.

Table 5.1: Chlorophyll *a*-normalized carbohydrate production (y=ax+b) during the daytime emersion period, 20 May 1999 (n.s.=not significant)

Depth (µm)	a (h^{-1})	b	R^2	p
Water-extractable carbohydrates				
0-200	19.9	7.2	0.74	<0.001
200-400	7.6	12.7	0.59	<0.001
400-600	5.6	19.1	0.37	<0.001
600-800	3.4	23.9	0.23	<0.05
800-1000	2.2	24.3	0.17	<0.05
1000-1500	1.2	31.8	0.03	n.s.
1500-2000	-0.5	40.7	0.03	n.s.
EDTA-extractable carbohydrates				
0-200	1.2	13.2	0.44	<0.001
200-400	1.6	16.8	0.09	n.s.
400-600	0.8	22.0	0.12	n.s.
600-800	0.4	31.1	0.007	n.s.
800-1000	0.05	33.5	0.0002	n.s.
1000-1500	-0.1	35.0	0.0008	n.s.
1500-2000	-3.2	49.0	0.12	n.s.

Table 5.2: Size distribution of water- and EDTA-extractable carbohydrates at three depth intervals at the start and end of the daytime exposure period, 20 May 1999. The values indicate the carbohydrate contents in $\mu g \cdot g^{-1}$, while values in brackets give percentages of the whole fraction. (n.d.: not detected).

fraction	Depth (µm)	Size class (kD)							
		>100		100-50		50-10		<10	
		Start	end	start	end	start	end	start	end
water-extr.	0-200	836 (29)	4774 (24)	375 (13)	1719 (9)	173 (6)	3819 (20)	1529 (53)	8975 (47)
	600-800	413 (25)	523 (21)	314 (19)	323 (13)	132 (8)	124 (5)	793 (48)	1518 (61)
	1500-2000	477 (37)	465 (34)	115 (9)	123 (9)	90 (7)	82 (6)	605 (47)	698 (51)
EDTA-extr.	0-200	441 (22)	512 (18)	180 (9)	199 (7)	260 (13)	114 (4)	1142 (57)	2019 (71)
	600-800	n.d.	320 (17)	n.d.	301 (16)	n.d.	245 (13)	n.d.	1036 (55)
	1500-2000	419 (22)	295 (22)	190 (10)	147 (11)	190 (10)	268 (20)	1104 (58)	643 (48)

Size distribution

Table 5.2 gives the results for the size distribution analyses. The water- and EDTA-extractable fractions showed a comparable size distribution. Both fractions contained high amounts of carbohydrates <10kD (47-71%), while carbohydrates in the size classes 100-50 and 50-10 kD were less abundant. During the emersion period, the relative contribution of the >100kD size class decreased slightly compared to the other size classes (t-test, $p<0.05$). The size distribution of the carbohydrate fractions did not vary with depth.

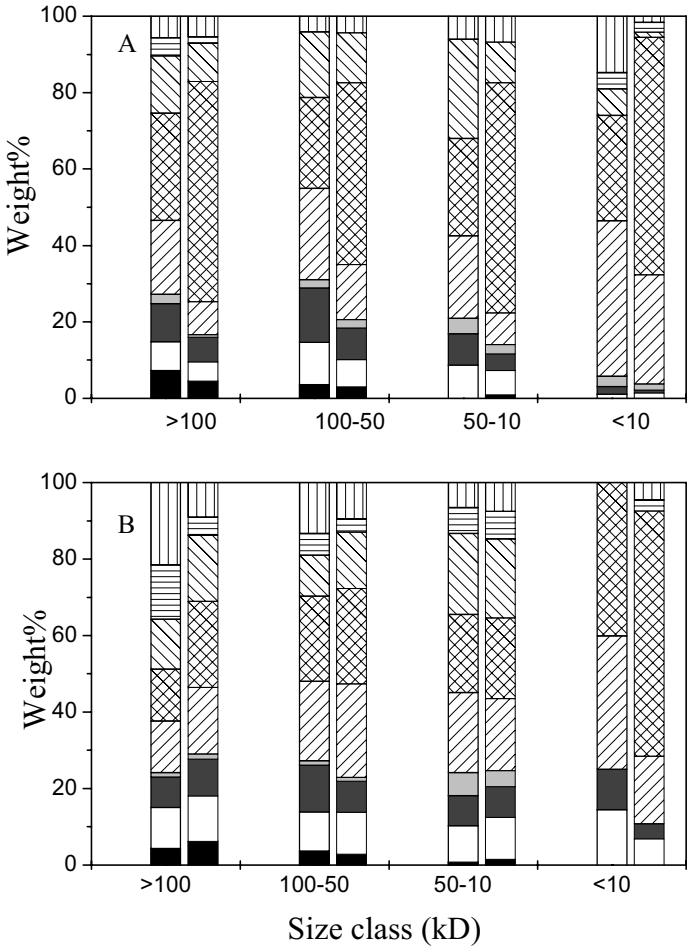

Figure 5.7. Relative monosaccharide composition (depth averaged) in different size fractions. Samples were taken during the daytime emersion period, 20 May 1999. (A); colloidal carbohydrates, (B); EDTA-extractable carbohydrates. For each size class left side bars represent the monosaccharide composition at the start of the emersion period while right side bars represent the monosaccharide composition at the end of the emersion period. ■: Fucose; □: Rhamnose; ■: Arabinose; ■: Glucose-amine; ▨: Galactose; ▩: Glucose; ▧: Xylose/Mannose; ▤: Galacturonic acid; ▥: Glucuronic acid.

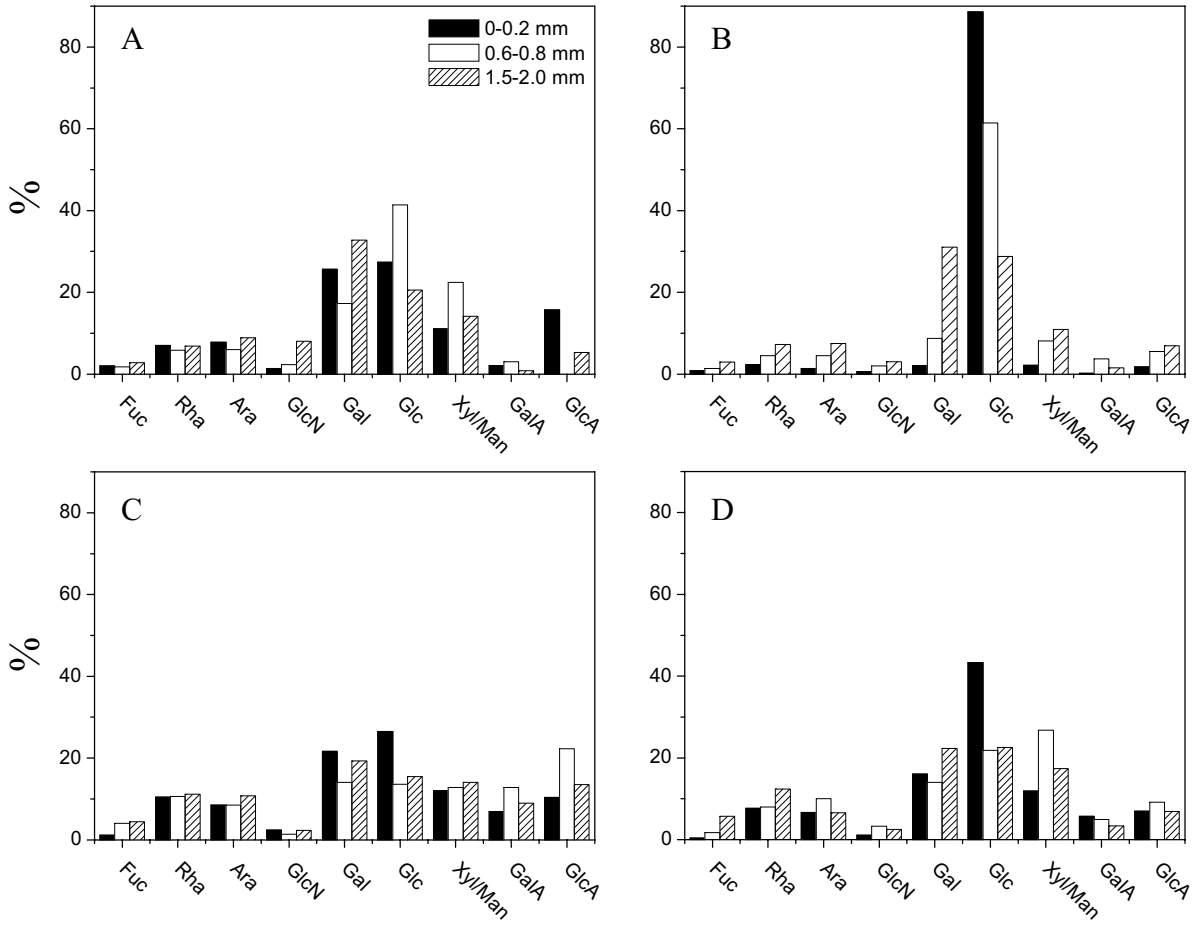

Figure 5.8. Relative monosaccharide composition at the three sampled depth intervals, during the daytime emersion period on 20 May 1999. (A); water-extractable carbohydrates, start of the emersion period, (B); water-extractable carbohydrates, end of the emersion period, (C); EDTA-extractable carbohydrates, start of the emersion period, (D); EDTA-extractable carbohydrates, end of the emersion period. Fuc=fucose; Rha=rhamnose; Ara=arabinose; GlcN=glucose-amine; Gal=galactose; Glc=glucose; Xyl/Man= xylose/mannose; GalA=galacturonic acid; GlcA=glucuronic acid.

Monosaccharide composition

The depth averaged monosaccharide compositions for the different size classes of carbohydrates are shown in Fig. 5.7. When the size fractions per sample are compared in terms of monosaccharide composition size classes >100, 100-50 and 50-10 kD showed no differences. However, the water-extractable carbohydrates <10 kD were enriched in galactose and depleted in xylose/mannose, rhamnose and arabinose when compared to the larger polymers. EDTA-extractable carbohydrates <10 kD were enriched in glucose while the contribution of xylose and mannose dropped to zero. Furthermore, the relative contribution of fucose decreased with decreasing size class in both carbohydrate fractions. The EDTA-

extractable carbohydrates contained at least twice as much uronic acids as the water-extractable fraction, regardless the size, except in the <10 kD size class.

The results in Fig. 5.7A show changes in the sugar composition of water-extractable carbohydrates during the emersion period. In all size classes the relative contribution of glucose increased about twofold by the end of the emersion period. Hence, the extracellular carbohydrates produced during emersion were relatively rich in glucose. The EDTA-extractable fraction >10 kD did not change composition during the emersion, whilst some variation in the <10 kD fraction was apparent.

The composition of the carbohydrates at different depth intervals is shown in Fig. 5.8. At the end of the emersion period glucose represented up to 85% of the sugars in the water-extractable fraction in the top 0.2 mm of the sediment. The contribution of glucose decreased rapidly with depth. At 1.5-2 mm, the contribution of glucose was only 28%, while galactose became the most abundant monomer. In the EDTA-extractable fraction, the contribution of glucose increased during emersion, although this was much less pronounced than for the water-extractable fraction. Again, most of this increase was associated with the surface 0.2 mm of the sediment.

Discussion

Carbohydrate dynamics

In this study, the dynamics of water- and EDTA-extractable carbohydrate fractions in an intertidal mudflat were investigated. Measurements of carbohydrate contents in the presence and absence of diatom biofilm structures revealed different dynamics between the two carbohydrate fractions. This was clear at the time scale of a tidal cycle but also when comparing the situation with and without a diatom biofilm. In June 1998, water-extractable carbohydrates that have been suggested to be indicative of microphytobenthic production (Underwood and Smith 1998a) were present in low amounts when compared to EDTA-extractable carbohydrates. No production of extracellular carbohydrates was observed over the tidal cycle. In May 1999, the contents of water- as well as EDTA-extractable carbohydrate were considerably higher than in June 1998. Water-extractable carbohydrate content was a factor 8 higher while EDTA-extractable carbohydrate content had increased by a factor 2-3. In contrast to the June 1998 situation, net production of EPS (mainly water-extractable) was observed resulting in a tenfold increase in carbohydrates by the end of the daytime emersion period. This material had disappeared at the start of the nighttime emersion period. These data suggest that water-extractable carbohydrates are recently produced and disappear rapidly from

the system. This carbohydrate is either washed away during immersion (Smith & Underwood 1998, de Winder et al. 1999) or utilized by heterotrophic consumers (van Duyl et al. 1999; Middelburg et al. 2000). EDTA-extractable carbohydrates are less dynamic and represent a more conservative fraction in these sediments.

Although clear differences in dynamics of the two carbohydrates were observed, both the size distribution and the monosaccharide composition of the carbohydrate fractions >10kD were remarkably similar. The monosaccharide composition of the smallest size fraction was different from the larger fractions, galactose being most abundant in the water-extractable fraction while in the EDTA-extractable fraction glucose was the predominant sugar. Paterson (1986) showed that lyophilization may damage diatoms present in the sediment. Also Janse et al. (1996) observed that internal carbohydrates present in the cells of *Phaeocystis globasa* were released when cells were frozen. Therefore, we can not exclude that storage carbohydrates (e.g. chrysolaminaran MW: 3-4 kD) and other low molecular weight carbohydrates were present in the <10 kD fraction.

The similarities that were found in monosaccharide composition and size distribution of the two fractions may hint to a common source of these carbohydrates or a rapid utilization of the degradable part of the carbohydrates. Although both the composition and size of the two fractions were broadly similar, the uronic acids in the EDTA-extractable fraction were twice as abundant compared to the water-extractable fraction. Anionic sugars (sulfonic acids, uronic acids) are considered to be important for the adsorption of EPS to sediment particles (Decho 1990). Therefore, it is possible that EDTA-extractable carbohydrate originates from the same source and could be produced by the conversion of water-extractable carbohydrate, involving interactions with sediment particles and metal-ions (Decho 1994).

The production of carbohydrate was only evident in the light. It was confined to the top 1 mm and showed its highest rates of production in the top 0.2 mm. This correlated with diatom biomass, which was also concentrated in this top layer of the sediment during daytime emersion. This clearly emphasizes the importance of light, which is strongly attenuated in silty sediments (Kühl et al. 1994). De Winder et al. (1999) and Staats et al. (2000a) demonstrated that the production of extracellular carbohydrate by benthic diatoms was coupled to photosynthesis and our results support this conclusion. Smith and Underwood (1998) observed that the increase in carbohydrate content was associated with the vertical migration of diatoms. In our study vertical diatom migration occurred at the start and end of the daytime emersion period, as was judged from the coloration of the sediment. This was not directly coupled to changes in carbohydrate content. Therefore, the excretion of extracellular carbohydrates as part of the mechanism of diatom motility is probably small compared to that produced through unbalanced photosynthetic growth.

In the light, the chlorophyll-specific rate of production of water-extractable carbohydrate carbon ($g \cdot g^{-1}$) amounted to 8 h^{-1} in the top 0.2 mm of the sediment. Assuming

chlorophyll specific carbon-fixation rates for benthic diatoms of 20 h^{-1} (Blanchard and Montagna 1992), we calculate that about 40 % of the carbon fixed may be excreted as carbohydrates. This number was experimentally shown by Middelburg et al. (2000), using *in situ* enrichments of the stable carbon isotope ^{13}C. However, the assumed rate of carbon fixation was taken from a subtropical mudflat. Blanchard et al. (1997) calculated a much lower rate of 11 h^{-1} in a temperate mudflat. Using this lower number we calculated that as much as 73% of fixed carbon might be excreted as carbohydrates. Using incubations with the radioisotope ^{14}C, Goto et al. (1999) reported that 42-73% of fixed carbon was excreted which confirms our estimates. As this extracellular carbohydrate only forms in the water-extractable fraction, it follows that the vast majority of this material disappears rapidly. Whether these carbohydrates dissolve in the water column or are degraded in the sediment remains to be investigated.

The major component of the EPS produced during the emersion period was glucose, which represented about 90% of the total. This was the case for carbohydrates of all size classes, indicating that the polysaccharides produced are highly variable in size or that the polymers are rapidly hydrolyzed into smaller molecules. Glucose is a preferred substrate for many heterotrophic consumers such as bacteria (Sawyer & King 1993). The first step in polymer degradation is its hydrolysis to molecules smaller than 0.6 kD (Weiss et al. 1991) by exo-enzymes. King (1986) and van Duyl et al. (1999) measured the activity of ß-glucosidases in intertidal mudflats and they demonstrated that benthic heterotrophic bacterial production depended on water-extractable carbohydrates in a diatom biofilm dominated mudflat. Aluhiware & Repeta (1999) found that the EPS excreted by three phytoplankton species were subject to a rapid partial degradation. The residual polysaccharides that remained resembled metabolically stable acyl heteropolysaccharides (Aluhiware et al. 1997) that accumulate in seawater. Our results are very similar. At the start of the emersion period and deeper in the sediment the contribution of glucose was considerably lower compared to the EPS that was excreted during the emersion period. This indicates that glucose is preferentially removed from the system. Therefore, EPS that was present at the start of the emersion period and deeper in the sediment seem to represent a polysaccharide fraction that is more resistant to microbial degradation.

Diatom migration

In June 1998, the sediment chlorophyll *a* content was low (<13 $\mu g \cdot g^{-1}$) and no clear pattern in the vertical distribution over a tidal emersion period was observed. This was probably the result of the low biomass and a high spatial variation. In May 1999, the chlorophyll *a* content ranged up to 150 $\mu g \cdot g^{-1}$ in the surface 0.2 mm during the daytime emersion. During this period, the majority of the diatoms were present in the surface sediment

layer to allow for maximum rate of photosynthesis. During nighttime emersion, microphytobenthos biomass was equally distributed in the upper 2 mm (Fig. 5.4) because the diatom had migrated deeper into the sediment. The upward migration towards the sediment surface is probably triggered by light, while in the dark the direction of movement of the diatoms may be random resulting in a homogeneous distribution. Alternatively, diatoms may sense chemical gradients that trigger the direction of migration.

Diatom migration occurs at the start and end of emersion periods as was shown by Serôdio et al. (1997) and Smith & Underwood (1998). In our study, this migration of diatoms was also observed. During the daytime emersion period the coloration of the sediment surface changed 30 min after the site became exposed and again 1 h before immersion. Remarkably, this was not reflected in the vertical profiles of chlorophyll *a*, which remained unchanged over the whole emersion period. This means that the migration of diatoms at the time that the sediment changed color was only within the top 0.2 mm (see also Wiltshire et al. 1998) Therefore, the differences in chlorophyll *a*-distribution between the day- and nighttime emersion period must have emerged during tidal immersion.

Guarini et al. (2000) described that microphythobenthic biomass is subject to short-term cyclic variations. In their study biomass production occurred in the light while losses were due to natural mortality, grazing and resuspension. Our results showed that microphytobenthic biomass did not increase over the course of the light period. This suggests that fixed carbon was not used for balanced growth of the organisms but predominantly excreted as extracellular carbohydrates (see above). During tidal immersion 23 % of the microphytobenthic biomass was lost, probably because of resuspension and migration to below the 2 mm zone.

Summary

In this study, the dynamics of two extracellular carbohydrate fractions were examined. Biochemically, both fractions had a similar composition. However, they behaved differently and ecologically these fractions have different impacts. The water-extractable carbohydrates represent a fraction that is highly dynamic. EPS produced during tidal emersion is rich in glucose (about 90%) and it disappears rapidly. It is turned over in the sediment (particularly the glucose part) but may also be washed out of the sediment during tidal immersion. The EDTA-extractable fraction is enriched in uronic acids and is associated with sediment particles. This fraction behaved more conservatively. EPS-production was strongly light dependent and associated with the presence of diatoms in the uppermost surface layer. During the nighttime emersion period no EPS production was observed and the diatom distribution did not vary over the depth investigated. About 40-73% of the carbon fixed during the light period was converted to extracellular carbohydrates, that are rapidly removed from the system

and therefore are likely to represent an important labile organic carbon pool in intertidal sediments.

CHAPTER 6

INTERPLAY BETWEEN BIOLOGY AND SEDIMENTOLOGY IN A MUDFLAT (BIEZELINGSE HAM, WESTERSCHELDE, THE NETHERLANDS)

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Abstract

The aim of this research was to investigate the importance of biological processes on the sediment characteristics and the morphology of a mudflat in the Westerschelde (The Netherlands). For this purpose, a transect in the Biezelingse Ham mudflat was sampled on a monthly basis. In spring, the muddy part of the mudflat was dominated by a biofilm of microphytobenthos that altered the morphology of the mudflat and resulted in a two-fold increase in sediment stability. The biofilm also bound fine grained sediment that was deposited. From June onwards, wind generated waves dominated the conditions at the mudflat which resulted in the disappearance of diatom biofilms, and caused a gradual erosion of the mudflat. During this period, meio- and macrofauna densities increased from which it was concluded that the hydrodynamic forces did not have a big impact on these communities. Spatial variations in sediment characteristics, morphology and biology were observed between station 1 and 2 on the one hand and station 3 on the other hand. This resulted in different responses to the changing conditions on the mudflat. In general, the results from this field study indicate that sedimentology and biology, interact in a complex manner with the hydrodynamic regime on a temporal as well as on a spatial scale.

Introduction

Sediment transport at intertidal mudflats is governed by physical, chemical and biological processes. Physical processes determine the magnitude of sediment transport to or from a mudflat, and are driven by tidal currents (Postma, 1961; Bell et al., 1997) and wind generated waves (Postma, 1957; de Jonge and van Beusekom, 1995; Bell et al., 1997). The organisms that inhabit intertidal mudflats may modify the structure of the sediment surface, which eventually changes the erosion threshold.

The surface of mudflats often supports communities of benthic diatoms. These organisms excrete large amounts of extracellular polymeric substances (EPS), which are known to be involved in vertical migration (Paterson 1989; Smith & Underwood 1998), or may result from overflow metabolism, due to unbalanced growth (Ruddy et al. 1998a, b). EPS consists mainly of polysaccharides (Hoagland et al. 1993). It was observed both in the laboratory experiments (Holland et al. 1974; Sutherland et al. 1998) as well as in field studies (de Boer 1981; Grant et al. 1986; Kornman & de Deckere 1998) that EPS may stabilize sediment surfaces.

Other organisms may also stabilize sediments. These include cyanobacteria, which mainly occupy sandy intertidal areas (Yallop et al. 1994) or heterotrophic bacteria (Dade et al. 1990). However, the mechanisms of stabilization may be different. Also meio- and macrofauna may be important in sediment transport processes. Both groups of organisms produce EPS and are in principle able to stabilize the sediment (Riemann & Schrage 1978; Meadows et al. 1990). However, they can also destabilize the sediment by grazing on microphytobenthos (Gerdol & Hughes 1994; Admiraal et al. 1983). The effect of meio- and macrofauna on sediment (de)stabilization is therefore complex. In some cases both stabilization and destabilization of the sediment by a single species was observed. For instance, Gerdol and Hughes (1994) found that the amphipod *Corophium volutator* caused destabilization of the sediment bed due to grazing on microphytobenthos and reworking of the sediment by burrowing and tube formation. In contrast, Mouritsen et al. (1998) attributed the stabilization of well defined bed structures to the presence of *Corophium volutator*. They suggested that the coating of the walls of burrowing holes by EPS was responsible for the observed stabilization (see also Meadows et al. 1990).

The aim of this research was to investigate the importance of biological processes for mudflat morphology and sediment characteristics and to identify interactions between the biology, sediment characteristics and the hydrodynamic regime acting on the mudflat. For this purpose the temporal variation of a broad set of biological and sedimentological parameters was measured monthly from March to September 1998. Measurements were carried out along a transect in seaward direction, thereby including stations with differences in sediment properties. The results indicate that sediment characteristics, mudflat morphology,

hydrodynamic regime and biology are interrelated in a complex manner. Furthermore, it was shown that also the sediment properties influence the biology in the sediment.

Material and methods

Site description

The study site was the Biezelingse Ham. This intertidal mudflat is situated in the Westerschelde in the south-western part of the Netherlands (Fig. 6.1). It has an area of about 1.5 km^2 and the mean tidal range is approximately 4 m. The Biezelingse Ham is a more or less protected embayment shielded by other mudflats on the east side. A freshwater inlet and a small saltmarsh are situated at the north side of the mudflat. At the mudflat some small drainage channels are present. The mudflat surface is mostly planar and major bedforms are generally absent. A transect (approximately 500 m in length) was established at the south side of the mudflat, running perpendicular to the low water line. Along this transect 3 sampling stations were chosen. Station 1 and 2 (resp. 50 and 250 m from the shore) consisted of muddy

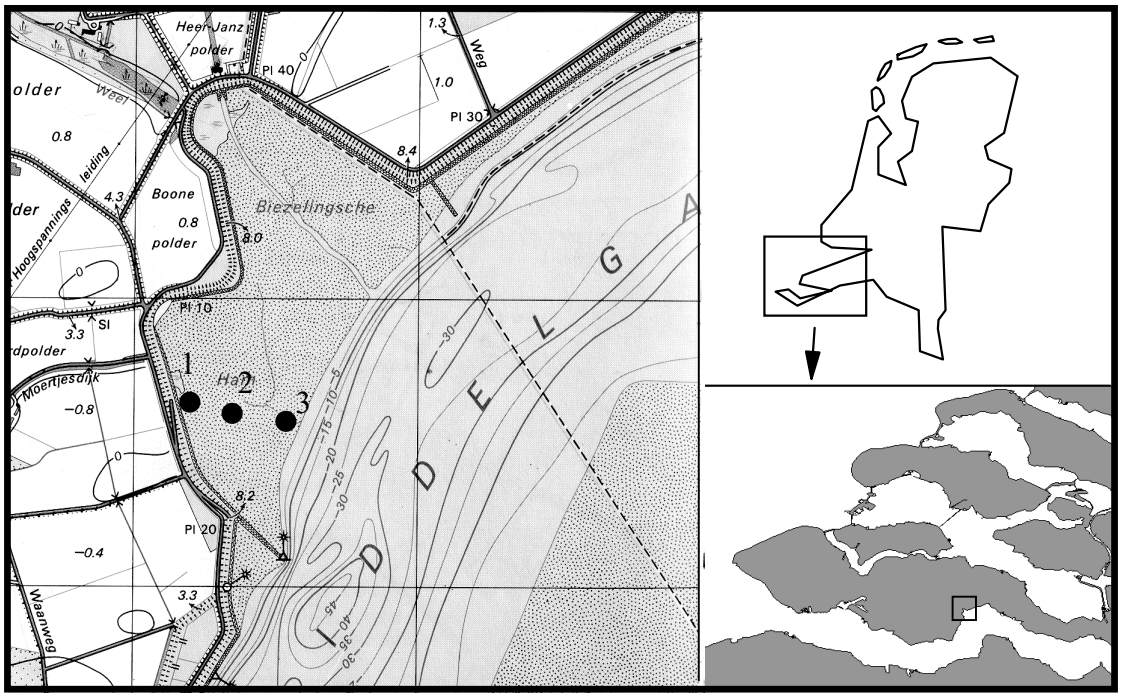

Figure 6.1.The Biezelingse Ham mudflat in the Westerschelde showing the location of the transect.

sediments (90%<63µm), while station 3 (450 m from the shore) was more sandy (60%<63µm). Between station 1 and station 2 there was a channel running parallel to the shore. The depression caused by the channel resulted in the tide coming in predominantly from the south side. As a result of the relief at this part of the mudflat and the direction of the incoming tide the three sampling stations had similar emersion times. Sampling was carried out monthly from March to September 1998.

Morphology

The changes in mudflat morphology at the sampling site were documented by photography and by describing the visual morphological features.

Sampling

Sediment was sampled for analysis of carbohydrates, chlorophyll *a*, meiofauna and macrofauna (n=3). For carbohydrate and chlorophyll *a* measurements, sediment was sampled using stainless steel cores (∅ 18 mm) which were sectioned in 2 parts: a surface sample (0-1 cm) and the layer of 1-5 cm. Meiofauna was sampled using perspex cores (∅ 36 mm) and also subdivided in 0-1 and 1-5 cm layers. Macrofauna was sampled from the top 30 cm using perspex cores (∅ 80 mm). The samples for meiofauna counting were fixed in 4% formaldehyde buffered with Borax. The samples for chlorophyll *a* and carbohydrate analyses were frozen at –20°C and subsequently freeze dried prior to analysis.

Carbohydrate analysis

Carbohydrates were determined using the phenol-sulfuric acid assay (Dubois et al. 1956). Carbohydrates were extracted from the sediment according to the following procedure. About 200 mg of freeze dried sediment was extracted with 1 ml of distilled water for 1 h at 30°C. The sample was centrifuged for 5 min at 6000×g. The supernatant contained the water-extractable carbohydrates. Subsequently, the pellet was extracted for 4 h with 1.5 ml of 0.1 M Na_2-EDTA at 20°C. The supernatant contained the EDTA-extractable fraction. The high molecular fraction of the water-extractable carbohydates was determined by precipitation in 80% (v/v) ethanol for 16 h at –20°C.

Chlorophyll a analysis

For pigment analysis, freeze dried sediment samples were ground and sonicated in 90:10 methanol : NH_4Ac for 5 min. Subsequently, the samples were centrifuged for 10 min at

1000×g. The extracts were analyzed by HPLC following the procedure described by Barranguet et al. (1997).

Meio- and macrofauna

Meiofauna was sieved over a 38 µm mesh sieve and counted using a disection microscope. Macrofauna was sieved over a 500 µm mesh sieve and subsequently fixed in 4% buffered formaldehyde. Species were identified, and counted using a disection microscope.

Sedimentology

The sediment characteristics that were measured included grain size, bed level, sediment stability, and water content (n=3). Grain size analysis was carried out on freeze-dried sediment samples of the 0-1 and 1-5 cm depth horizons of perspex cores (∅ 36 mm). Grain size distribution was determined using a Malvern particle sizer 3600 E operated with a 300 mm lens.

Water content of the upper 1 cm sediment layer was calculated from the wet and dry weight of the sediment.

The threshold velocity for erosion was measured using a Cohesive Strength Meter (Paterson 1989). Briefly, the system measures sediment resuspension in a test chamber filled with water and positioned on the sediment surface by applying a water jet at different velocities, perpendicular to the sediment surface. The point of incipient erosion (see also Yallop et al. 1994) was used as a relative measure for the stability of the surface sediment.

For bed level measurements, 3 plots were established per station. These plots consisted of 3 metal poles which were pushed into the sediment in a triangle. For bed level measurements, a triangular wooden plate was placed on top of the poles and the distance from the plate to the sediment surface was measured at nine points between the poles. The results from March were used as a reference point

Wind data

Data on windspeed and wind direction (10 min. interval) were obtained from the Ministry of Transport, Public Works and Water Management, and were measured at a measuring pole situated in the Westerschelde, 5 km east from the Biezelingse Ham. From these data, daily average windspeeds and wind directions were calculated for the period March – September 1998.

Statistical analyses

Spearman's rank correlation statistics were used to investigate the correlations between the various variables. One way ANOVA was performed on log transformed data to test changes along the transect. All statistics were performed using the Statistica 5.1 package.

Results

Morphology

At the start of the sampling campaign in March 1998 the morphology at the three stations along the transect showed marked differences. Station 1 was muddy and was characterized by a homogeneous structure and a wet surface layer (Fig. 6.2A). Station 2 was

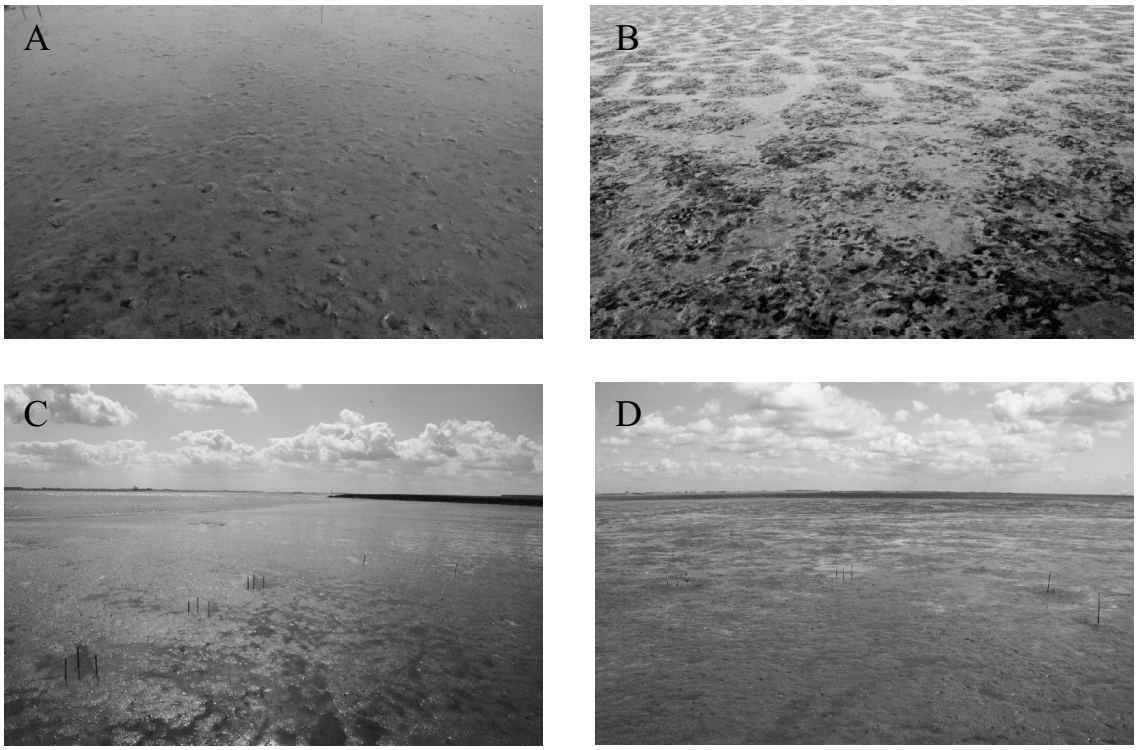

Figure 6.2. Photographs of morphological features in de Biezelingse Ham mudflat. Homogeneous mudflat structure at station 1, April 1998 (A); criss-cross structure with diatom biofilms at station 2, May 1998 (B); typical morphology at station 3, August 1998 (C); structure of randomly distributed elevated parts and depressions at station 2, August 1998 (D).

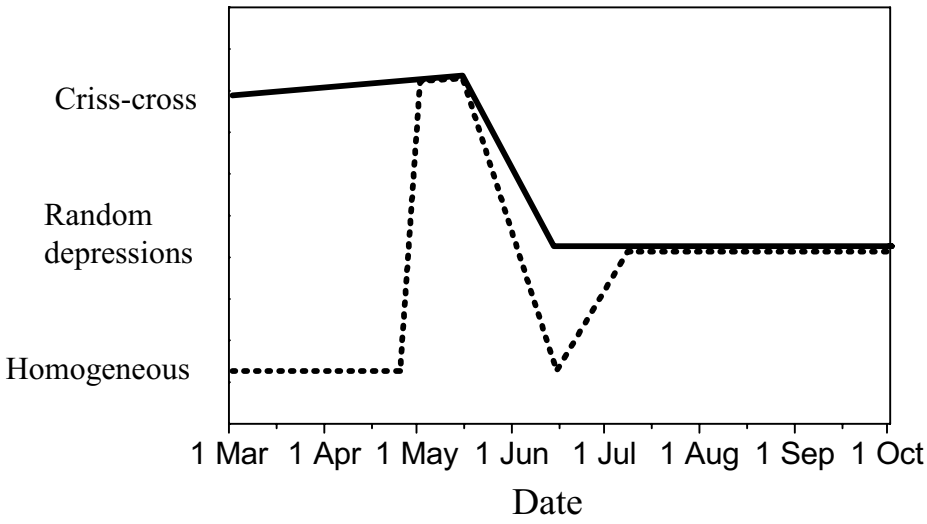

Figure 6.3. Schematic representation of changes in morphology at station 1 (···) and station 2 (—) in the period from March to September 1998.

characterized by a so called criss-cross pattern with regularly spaced elevated and lowered parts (Fig. 6.2B). Station 3 was situated close to the low water line and the sediment at this part of the mudflat contained more sand. The morphology showed a mosaic of elevated parts and depressions, frequently accompagnied by erosion features on top of which small patches of diatoms were often visible (Fig. 6.2C). The sediment morphology of station 1 and 2 changed during the study period. This is schematically depicted in Fig. 6.3. During the period from March to May, diatom abundance increased steadily at station 2, which coincided with the criss-cross pattern. In May, a dense diatom mat formed at station 1. During this month, mudflat morphology changed rapidly from a homogeneous structure to a criss-cross pattern. In June, after a period of more severe weather conditions, diatoms disappeared, as did the criss-cross features. From July onwards, both station 1 and 2 possessed an identical surface morphology and were characterized by randomly distributed elevated parts and depressions (Fig. 6.2D). The mudflat morphology of station 3 did not change much throughout the sampling period although in May increased patches of diatoms were observed.

Biology

Chlorophyll *a* contents as well as water- and EDTA-extractable carbohydrate contents are shown in Fig. 6.4. At station 1 and 2 chlorophyll *a* and carbohydrate contents followed largely the same pattern. In May there was a sharp increase in both chlorophyll *a* and carbohydrate contents at station 1. At station 2 chlorophyll *a* and carbohydrate contents were elevated in the period of March to May with the highest contents occurring in May. From

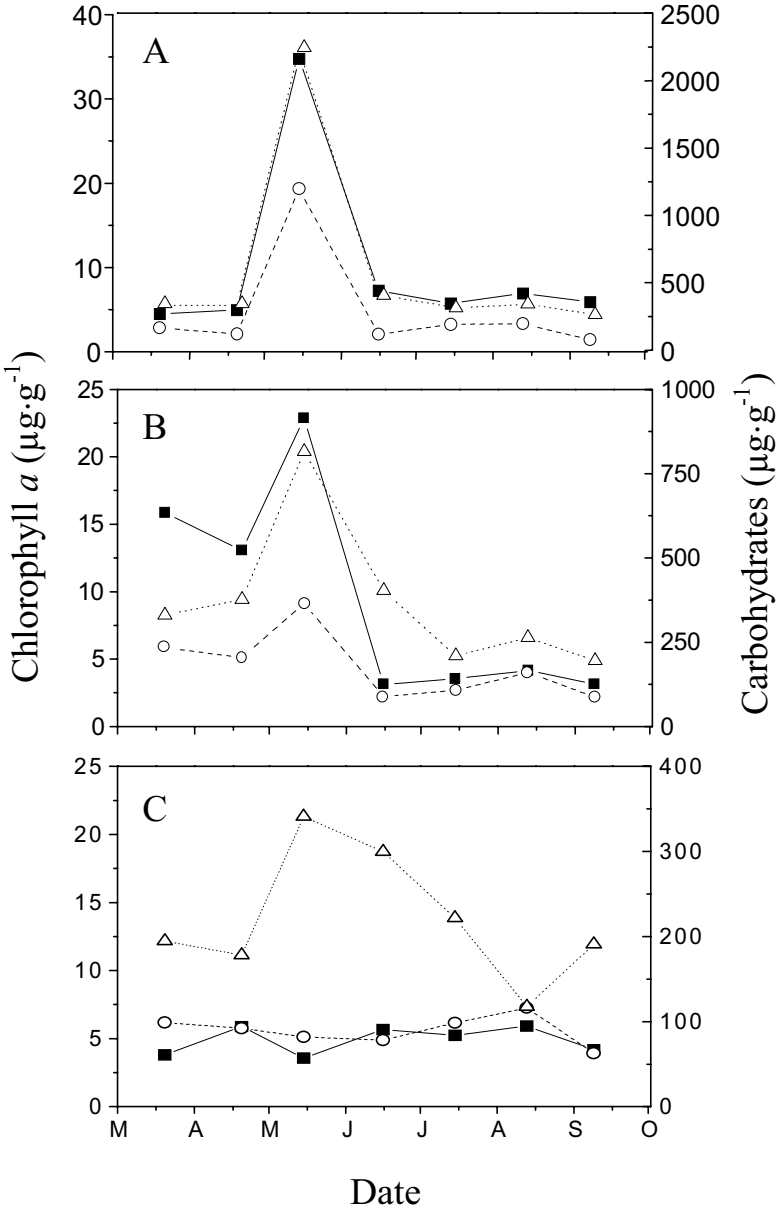

Figure 6.4. Chlorophyll *a* (■), water-extractable carbohydrate (o) and EDTA-extractable carbohydrate contents (∆) at station 1 (A), station 2 (B) and station 3 (C) from March to September 1998. Note different scales.

June onwards contents were generally lower than in the preceding 3 months. At station 3 chlorophyll *a* and water-extractable carbohydrate contents were constant throughout the sampling period. However, EDTA-extractable carbohydrate contents showed a sharp increase in May, subsequently decreasing gradually during the following months. The percentage of high molecular compounds in the water-extractable fraction varied considerably at all stations

Table 6.1. Spearman Rank correlation coefficients between chlorophyll *a*, carbohydrate contents (CHO), stability measurements, median grain size and bed level. Data are from station 1, 2 and 3 over the period March-September 1998 (n=63). When correlations are significant Spearman Rank coefficients are given between brackets. n.s., not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

	Chl. *a*	Water-extr. CHO	EDTA-extr. CHO	Stability	Median grainsize
water-extr.	*** (0.68)				
EDTA-extr.	n.s.	** (0.56)			
Stability	n.s.	n.s.	n.s.		
Median grainsize	* (-0.51)	*** (-0.73)	*** (-0.81)	n.s.	
Bed level	n.s.	n.s.	n.s.	n.s.	n.s.

and ranged from 20% in the presence of diatom films to values of 60% when diatoms were absent (data not shown). Statistical analyses of the data of chlorophyll *a*, and the two carbohydrate fractions showed that carbohydrate contents were statistically different along the transect (One way ANOVA, water-extractable carbohydrates, $F_{(2, 58)}$=10.88, p<0.001; EDTA-

Table 6.2. Spearman Rank correlation coefficients between chlorophyll *a*, carbohydrate contents (CHO), stability measurements, median grain size and bed level. Data are from station 1 and 2 over the period March-September 1998 (n=42). When correlations are significant Spearman Rank coefficients are given between brackets. n.s., not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

	Chl. *a*	Water-extr. CHO	EDTA-extr. CHO	Stability	Median grainsize
Water-extr.	*** (0.83)				
EDTA-extr.	* (0.57)	n.s.			
Stability	n.s.	n.s.	n.s.		
Median grainsize	n.s.	* (-0.56)	** (-0.72)	n.s.	
Bed level	** (0.67)	** (0.71)	*** (0.83)	n.s.	*** (-0.88)

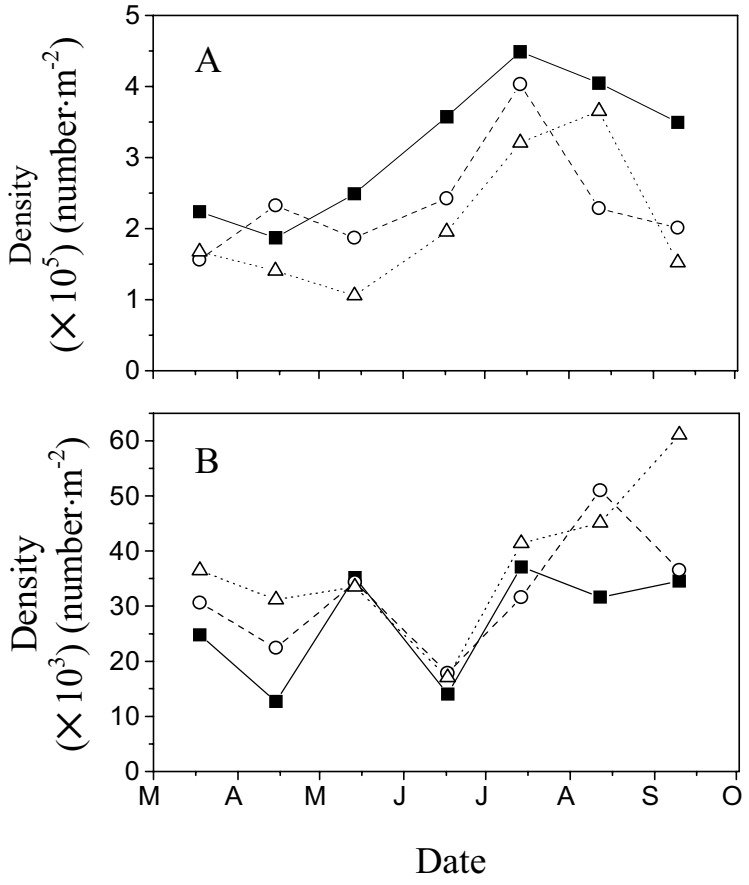

Figure 6.5. Meiofauna density (A) and macrofauna density (B) at station 1 (■), station 2 (o) and station 3 (Δ) in the Biezelingse Ham mudflat from March to September 1998.

extractable carbohydrates, $F_{(2\ ,\ 58)}$=9.85, p<0.001) whereas chlorophyll *a* contents were not significantly different (One way ANOVA, $F_{(2\ ,\ 58)}$=2.33, p=0.13) . Chlorophyll *a* contents correlated well with water-extractable carbohydrates (Table 6.1). However, EDTA-extractable carbohydrates did not correlate with chlorophyll *a* when taking all data into account. When the data of station 3 were excluded from the dataset correlation with chlorophyll *a* was significant at p<0.05 (Table 6.2).

Meiofauna densities showed similar variations for all stations, although the numbers decreased slightly along the transect (Fig. 6.5A). Densities increased from April to July and decreased again from August onwards, which was probably caused by grazing by macrofauna. The variations of the macrofauna community were comparable for all stations along the transect (Fig. 6.5B). There was an increase in May followed by a sudden sharp decrease in June, which coincided with an average erosion of about 2 cm of sediment due to a change in weather conditions in the beginning of June. A major part of the macrobenthos that

Table 6.3. Commonly occurring macrobenthos species at station 1, 2 and 3 in the Biezelingse Ham mudflat. For each species the minimum and maximal number of individuals in the period March to September are indicated.

Species	Station 1	Station 2	Station 3
	Density (number m^{-2})		
Arenicola marina	0	0	0-166
Cerastoderma edule	0-597	66-1061	0-13926
Corophium volutator	5570-20889	199-20955	0-21950
Heteromastus filiformis	4178-7494	7692-15385	7629-12799
Hydrobia ulvae	332-6499	729-6234	862-4841
Macoma balthica	0	0	464-1192
Manayunkia aestuarina	0-7228	0	0
Nereis succinea	862-4112	663-4377	0-1989
Pygospio elegans	66-3913	66-3714	663-5239
Tharyx marioni	0	0-1326	862-13926

lives in the surface layer of the sediment was probably removed as a result of these changing conditions. This was also supported by the distribution of *Corophium volutator*, which was abundant in May only at station 1. In June this organism was present in low numbers both at stations 1 and 2 (data not shown). This pattern was not observed for other macrofauna species and was attributed to the capacity of *Corophium volutator* to swim back to the sediment surface. From July onwards the macrobenthic community recovered rapidly and in September reached numbers of 40.000 to 60.000 individuals·m^{-2}. *Corophium volutator, Heteromastus filiformus* and *Hydrobia ulvae* occurred commonly throughout the entire transect and represented a major part of the total number of macrofauna (Table 6.3). Other species such as *Manayunkia marioni*, *Tharix marioni* and *Macoma balthica* were resticted to a much smaller area where they were locally abundant.

Sedimentology

Temporal variations in the treshold velocity for erosion and bed level are shown in Fig. 6.6. In the period of March to May, sediment stability increased and there was net deposition of sediment at all stations along the transect. From June onwards erosion took place at station 1 and 2 and reached a maximum of 5 cm at the latter. Bed level did not change significantly at station 3 during that period. In June, there was a sharp decrease in sediment stability at all

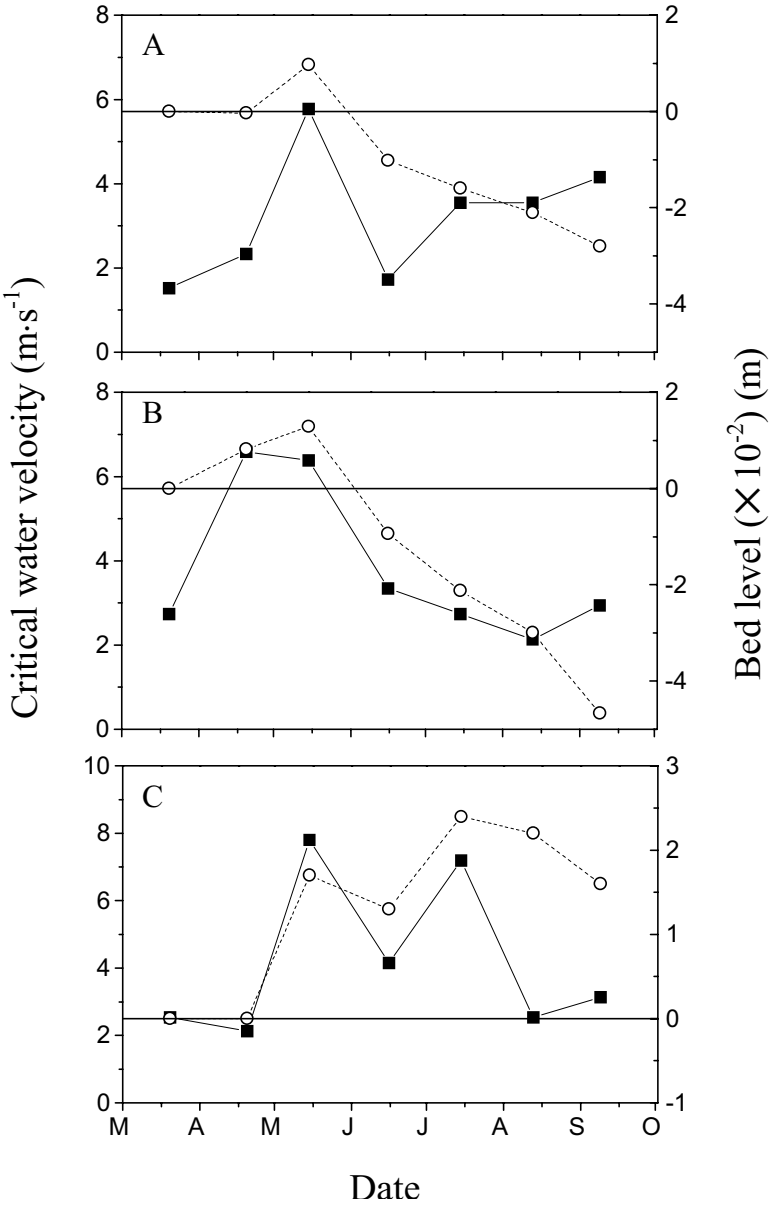

Figure 6.6. Sediment stability (■) and bed level (o) at station 1 (A), station 2 (B) and station 3 (C) from March to September 1998.

stations. At station 1, sediment stability recovered from July onwards while at station 2 stability remained low. Sediment stability at station 3 was higher from May to July when compared to before or after this period. When sediment stability is plotted as a function of carbohydrate content, it is clear that most data cluster together (Fig. 6.7A). The 3 points that do not cluster represent measurements done in the presence of dense diatom mats. At station 3, sediment stability was correlated with EDTA-extractable carbohydrates (R^2=0.51, $p<0.05$), while for water-extractable carbohydrates any correlation was absent (R^2=0.02) (Fig. 6.7B). When the stability data were merged, they did not show any correlation with either biological

or sedimentological variables (Table 6.1). Taking into account only data of stations 1 and 2 did not improve the correlation.

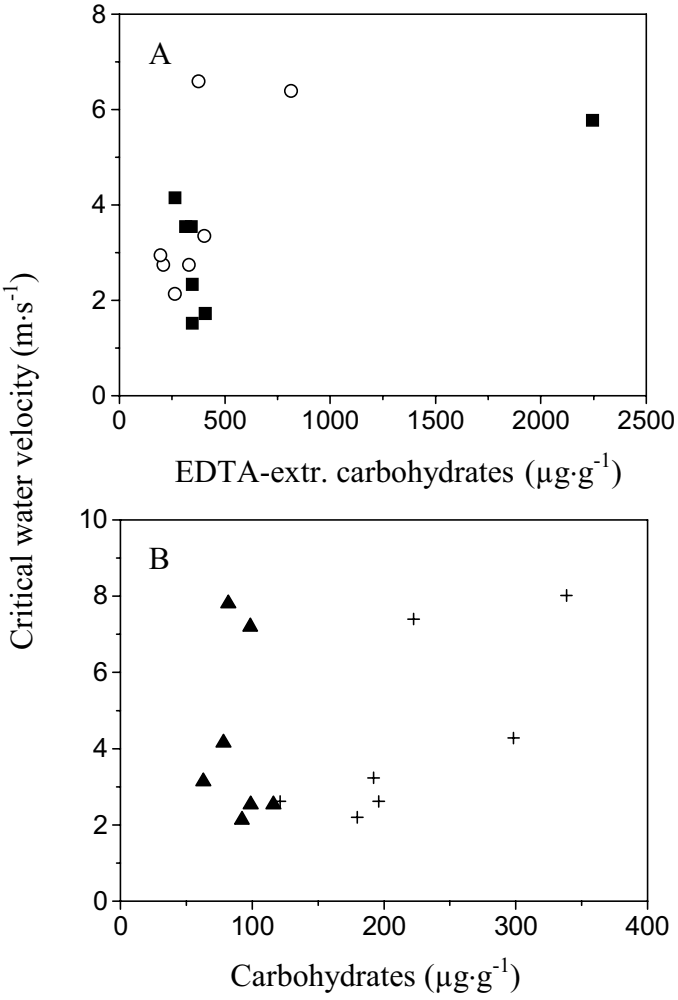

Figure 6.7. The influence of carbohydrate contents on sediment stability. The effect of EDTA-extractable carbohydrates at station 1 (■) and 2 (o) (A) and the effect of water- (▲) and EDTA-extractable carboydrates (+) at station 3 (B).

Water content in the upper 1 cm of the sediment showed a decreasing trend over the sampling period at all stations and varied between 0.41-0.77 for stations 1 and 2, and 0.38-0.57 for station 3. Typically, the highest values were observed in May in the presence of diatom mats. Median grain size values for station 1 and 2 were typically in the range of 15 to 50 µm while at station 3 sediment was coarser with a median grain size varying from 30 to 60 µm in the upper 1 cm. As is shown in Fig. 6.8 fine material was trapped on the mudflat

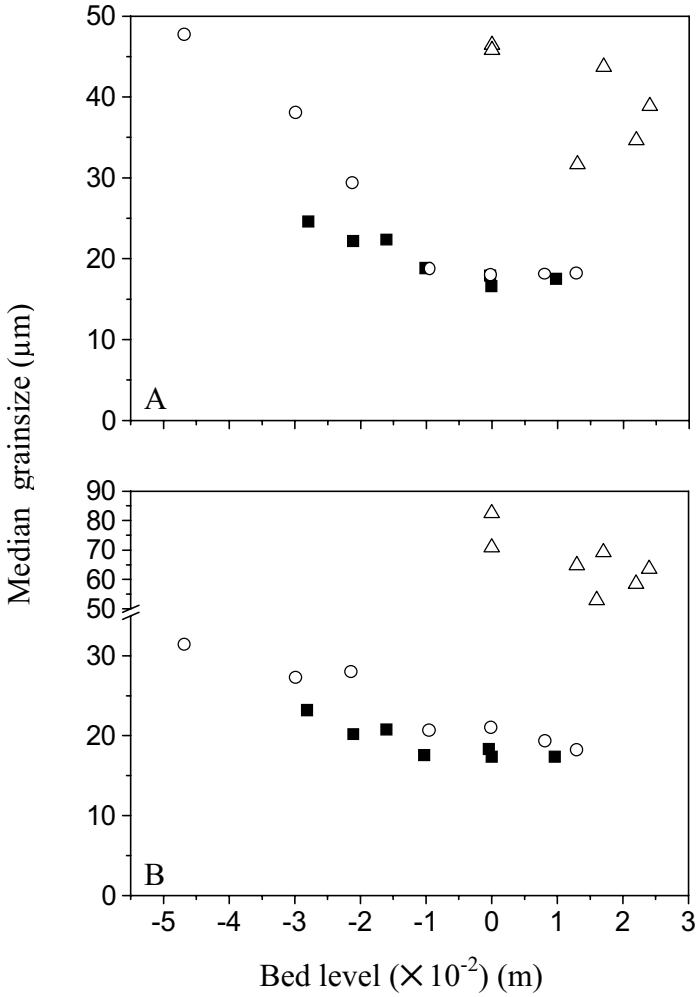

Figure 6.8. Median grain size at station 1 (■), station 2 (o) and station 3 (Δ) plotted against bed level. Median grain size was analyzed in the 0-1 cm sediment layer (A) and in the 1-5 cm sediment layer (B).

during deposition events. During erosion there was a discrimination between fine and coarse material, resulting in an increase of the median grain size (which was especially clear at station 2). At 1-5 cm depth there was a clear relation between grain size and bed level at all three stations (Fig. 6.8B). The median grain size along the transect is significantly correlated with water- and EDTA-extractable carbohydrate fractions ($p<0.001$) and with chlorophyll *a* ($p<0.05$). When taking all data into account, bed level did not show a significant correlation with any of the variables. However, when station 3 was excluded from the dataset bed level was highly significant with all variables except for stability (Table 6.2). This difference between stations 1 and 2 on the one hand and station 3 on the other hand was also reflected in Fig. 6.9. It shows that monthly changes in bed level were proportional to monthly stability changes, the regressions being different for the muddy and sandy sites.

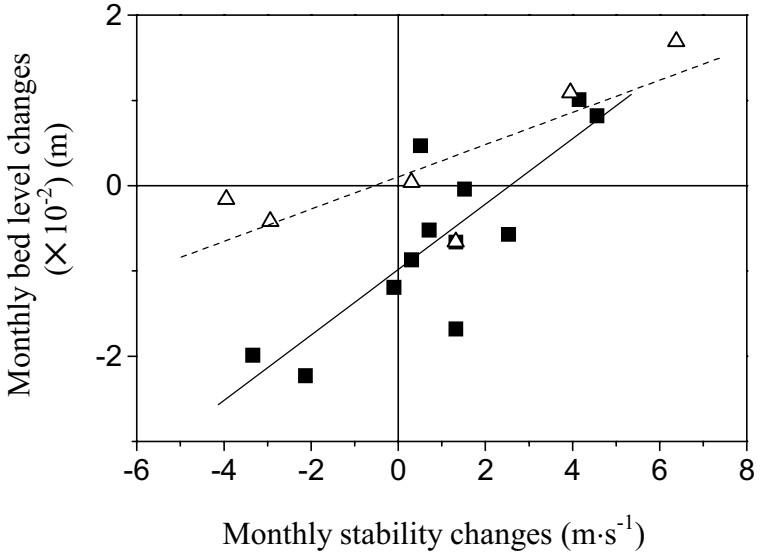

Figure 6.9. Comparison of monthly changes in stability and monthly changes in bed level for muddy (station 1 and 2) (■) and sandy (station 3) (Δ) sites.

Discussion

Over the sampling period clear temporal and spatial patterns in biology and sediment characteristics were observed. In the period from March to May there was a build up of microphytobenthos biomass at the stations 1 and 2, which resulted in the formation of dense diatom mats in May. Diatom mats are a common phenomenon of intertidal mudflats (Cadée and Hegeman 1974; Admiraal et al. 1982) and their effects on sediment characteristics, especially the stabilization of sediments has been described for muddy sediments (Underwood et al. 1993a; Kornman & de Deckere 1998) and sandy sediments (de Boer 1981; Grant et al. 1986; Vos et al. 1988). In this study, the influence of the microphytobenthos on sedimentary processes was evident from a number of observations. In spring the sediment morphology changed drastically, sediment stability increased and fine material was fixed to the diatom mats resulting in the net deposition of 1 cm of sediment on average (Fig. 6.6 and 6.8). Also the water content of the sediment was higher in the presence of diatom biofilms, which was attributed to the water retaining capacity of biofilms (Decho 1990).

Diatom blooms generally collapse after a period of a few weeks to a few months. Possible causes for this collapse may include i) grazing by macro- or meiobenthos (Admiraal et al. 1983; Gerdol & Hughes 1994) ii) nutrient limitation iii) dessication and iv) resuspension in the water column. The sudden decrease in diatom biomass in June 1998 coincided with a change in weather conditions by which the mudflat became exposed to westerly off shore

Table 6.4. Montly wind directions, average monthly windspeeds and maximum monthly windspeeds at the Westerschelde in the period March – September 1998.

Month	Wind direction											
	north - east (0-90°)			east - south (90-180°)			south - west (180-270°)			west - north (270-360°)		
	days	average (m/s)	max. (m/s)	days	average (m/s)	max. (m/s)	days	average (m/s)	max. (m/s)	days	average (m/s)	max. (m/s)
March	5	5.1	8.2	6	6.2	8.6	13	7.1	12.7	7	4.9	6.4
April	4	5.1	6.7	11	5.7	11.7	11	6.2	9.4	4	3.9	4.6
May	9	4.2	5.8	9	4.3	7.5	7	5.7	11.0	6	4.7	6.3
June	2	5.1	5.3	3	4.1	6.6	19	6.5	9.4	6	5.9	7.0
July	-	-	-	4	3.6	5.1	21	7.1	10.9	6	5.5	7.7
August	1	3.3	3.3	5	3.6	4.8	16	4.8	8.0	9	6.2	10.5
September	3	3.9	4.6	8	4.4	5.2	14	6.0	10.9	5	11.7	12.5

winds (Table 6.4). Although windspeeds were not exceptionally high in this period (4-9 $m \cdot s^{-1}$), rapid erosion of 2 cm of the sediment occurred , which caused the resuspension of the diatoms as well as a considerable part of the macrobenthos living in the top layer of the sediment. De Jonge & van Beusekom (1995) observed that at windspeeds > 3 $m \cdot s^{-1}$ resuspension by wind generated waves takes place. Wind generated waves are far more important for resuspension processes within the ranges of tidal currents and windspeeds. Therefore it seems likely that particularly during ebb tide, off shore winds resulted in erosion of material at stations 1 and 2.

In the period from the end of March to the beginning of June the mudflat was exposed predominantly to onshore easterly winds. In April and May short periods of strong westerly winds (8-11 $m \cdot s^{-1}$) occurred (Table 6.4), but they did not have a visible effect on the sediment morphology and diatom structures. Hence, it was concluded that diatom biofilms were efficient in stabilizing the sediment surface, and prevented erosion.

Sediment characteristics at station 3 were clearly different from station 1 and 2. Whereas station 1 and 2 consisted of muddy sediment with a high silt content, station 3 was more sandy. This type of sediment zonation is well described (Postma 1961) and is a result of differences in hydrodynamic conditions over the mudflat. Differences in sediment characteristics have an impact on the organisms living in these sediments. Brotas & Plante-Cuny (1998) found clear differences in the temporal patterns of pigments between muddy and sandy sites that corresponded well to the changes in chlorophyll *a* that were found in this study. Also Delgado et al. (1991) stated that resuspension of diatoms is directly dependent on sediment type. In the Biezelingse Ham different species of macrofauna were confined to

specific zones (Table 6.3). Some species showed a preference for sandy sediments (e.g. *Macoma balthica, Tharix marioni, Cerastoderma edule*) whereas others where confined to muddy sediments (e.g. *Manayunkia aestuarina, Nereis succinea*). Furthermore, at station 3 diatom biomass did not vary during the season, while dense mats were absent (see also Brotas & Plant-Cuny 1998). In addition, changes in bed level and sediment stability were different compared to the muddy sites (Fig. 6.6). This is also clear from Fig. 6.9, which shows that monthly bed level changes are proportional to monthly changes in sediment stability. The relationships were different for the muddy stations when compared to station 3. The relation between monthly bed level changes and monthly changes in stability were probably not the result of a direct correlation between the two parameters but it indicated that factors, which determine the monthly changes in bed level, act in a similar way on sediment stability. In this case high positive changes in stability and bed level change are a result of calm weather and the appearance of diatom films. Highly negative changes reflect erosion caused by high wave energy. All together this indicates that the processes taking place at station 3 were fundamentally different from those at the other stations.

Apart from the decrease in macrofauna density in June, macro- as well as meiofauna did not seem to be vulnerable to hydrodynamic forcing on the mudflat. This was probably the result of a rapid vertical migration, so that resuspension caused by erosion was prevented. Both meio- and macrofauna densities increased during summer, mostly independent of sediment characteristics. At station 3, high densities of *Cerastoderma edule* that were present in August and September possibly caused a decrease in sediment stability (Flach 1996).

The correlations between the different variables were examined using Spearman rank coefficients (Table 6.1 and 6.2). Water-extractable carbohydrates correlated significantly with chlorophyll *a* content and the complete data set of chlorophyll *a* and water-extractable carbohydrates agreed with the predictions formulated by Underwood & Smith (1998b). In that study, the conversion between water-extractable carbohydrates and the polymeric (EPS) fraction was done assuming a constant ratio of EPS to water-extractable carbohydrates of 0.2-0.3. In this study, ratios were between 0.2-0.6, with the lowest values recorded when diatom biofilms were present. In diatom cultures cell lysis occurs during freeze drying (unpublished observation) and this would have the risk of including endogenous carbohydrates in the water-extractable fraction. The low ratio during the diatom bloom suggests that cell lysis may also have occurred during freeze drying of the sediment samples.

Furthermore, carbohydrate concentrations were significantly related to median grain size, which is in agreement with previous observations (Bergamaschi et al. 1997). These workers suggested that attachment of carbohydrates to sediment particles is important in sediments (see also Taylor & Paterson 1998). This will have implications for the interactions of carbohydrates in sediments which are known to be complicated (Decho 1994). Grain size is important since it determines the number of active adsorption sites and thereby the degree of

interaction. When compared to mud and silt, sand grains are hardly charged and therefore a relatively small concentration of carbohydrates can be adsorbed to sandy sediments. Freshly produced carbohydrates therefore, are to a large extend non- or loosely associated and will wash away during immersion. In that case, the water-extractable carbohydrate fraction will be low and constant while EDTA-extractable carbohydrates are bound to the sediment, causing stabilization. In muddy sediments the adsorptive surface area is large, resulting in a broad range of associations and in higher carbohydrate contents. Due to the complexity of association in muddy sediments a large part of the loosely bound carbohydrates will be retained in the sediment, resulting in increased concentrations of water-extractable and EDTA-extractable carbohydrates (Fig. 6.3A, B).

From Spearman rank coefficients it was shown that sediment stability was not correlated to any of the variables included. In intertidal mudflats the influence of biology on the stabilization of sediment is thought to be mediated by the excretion of EPS particularly by benthic diatoms (Paterson 1989; Sutherland et al. 1998). At stations 1 and 2 a high sediment stability indeed coincided with elevated carbohydrate concentrations and a high diatom biomass. When diatom biofilms were absent sediment stability appeared to be governed by other factors than carbohydrate concentrations alone. At station 3, EDTA-extractable carbohydrates were correlated with stability. The origin of the EDTA-extractable carbohydrate fraction however, was not clear. The phototrophic community seemed to be unimportant because the biomass was continuously low. The results did not suggest a meio-or macrofaunal origin. Furthermore, it was reported that bacterial numbers are closely related to chlorophyll *a* (Cammen & Walker 1986; Cammen 1991). Therefore, a bacterial origin of the EPS seems unlikely.

When station 3 was excluded from the dataset bed level change was highly significant with all variables except for stability (Table 6.2). This again indicated that processes such as bed level change are highly dependent on the position on the mudflat and the hydrodynamic forces to which they are exposed.

In summary, sediment transport and the morphodynamics in the Biezelingse Ham was caused by a combination of biology and physical processes, which acted together in a complex way. When considering the effect of biology on sediment transport, diatom biofilm formation proved to be efficient in stabilizing the sediment and preventing sediment resuspension. However, above the threshold shear stress erosion takes place and biofilms disappear rapidly. Macro- and meiobenthos are less vulnerable to physical stress than diatoms and they did not appear to have a big impact on sediment processes. Finally, this study showed that spatial differences in sediment structure, even on short distances had a big effect on biological and physical processes.

CHAPTER 7

THE EFFECT OF GEOMORPHOLOGICAL STRUCTURES ON POTENTIAL BIOSTABILIZATION BY MICROPHYTOBENTHOS ON INTERTIDAL MUDFLATS

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Abstract

The chlorophyll *a* and water-extractable carbohydrate content of sediments were measured at Skeffling mudflat in the Humber estuary, UK, in July 1997 as part of a fieldwork experiment carried out within the framework of the INTRMUD project. The aim was to analyze the spatial variations of chlorophyll *a* and water-extractable carbohydrate concentrations within the surface 1 cm of sediment (together with physical variables) in the different macroscopic sedimentary structures found at four stations along a cross-shore transect. The underlying assumption was that epipelic microalgae (chlorophyll *a*) produce Extracellular Polymeric Substances (EPS) that are largely comprised of carbohydrates. This organic material binds sediment particles and thus contributes to enhance sediment cohesiveness and sediment stability. Therefore, the shape and the strength of the relationship between chlorophyll *a* and water-extractable carbohydrates are fundamental for assessing the role of autotrophic microbial communities in biostabilization processes. At station A, the highest level of the mudflat, there were no obvious sedimentary features, while a ridge (crest) and runnel (trough) system was present at mid-tidal stations (B and C). At station D, the sediment was sandier; crests and troughs were obvious but did not form a ridge and runnel system as at stations B and C.

Taking all data together, a significant positive linear relationship between water-extractable carbohydrates and chlorophyll *a* was found. When the data were analyzed separately by station there was no relationship between variables at the sandy station (D). At stations B and C, there was a difference in the chlorophyll *a*-carbohydrate relationship between ridges and runnels: (i) there was no relationship in runnels, i.e. carbohydrate concentration was roughly constant whatever the mud chlorophyll *a* content, and (ii) there was a positive linear relationship in ridges.

This indicates that the increase of epipelic biomass on ridges increases the amount of EPS, which is likely to stabilize the sediment surface of these features. The biomass level in runnels is lower and does not enhance the amount of EPS. Therefore, the activity of epipelic

microalgae in runnels does not contribute to sediment stability. This observed difference between ridges and runnels does not mean that epipelic microalgae from these two features necessary behave in a different way; carbohydrates produced by microalgae in runnels are very likely to be dissolved because of the higher water content. Thus epipelic algae cannot build up a pool of carbohydrates in runnels. As a conclusion, it is clear that geomorphological features of intertidal mudflats influence biological processes in a way which exacerbates the physical processes: (i) ridges are regularly exposed and the sediment surface is stabilized, which apparently favors microphytobenthos growth and carbohydrates production with a further increase in sediment stability (according to our initial assumption); (ii) runnels are drainage structures with a high water content, which prevents microphytobenthos from building up a carbohydrate pool. Therefore, there seems to be a synergistic effect between physical and biological processes on ridges to stabilize the sediment surface.

Introduction

Intertidal mudflats are a prominent geomorphological component of estuaries, and support populations that contribute a large part of their biological productivity. Generally, microphytobenthos is the most important primary producer in intertidal mudflats (Pinckney & Zingmark 1991; Cariou-Le Gall & Blanchard 1995). Not only does it supply the benthic foodweb with organic matter. Through its resuspension in the water column, microphytobenthos also fuels the planktonic foodweb (de Jonge & van Beusekom 1992, 1995). Because of this high productivity, and its potential effects on sediment properties through the production of Extracellular Polymeric Substances (EPS), microphytobenthos is likely to play a significant role in the biostabilization of the sediment surface, *i.e.* that part of sediment cohesiveness that can be attributed to biological activity (Paterson 1995, 1997).

Epipelic diatoms, which are the dominant group of microphytobenthos in intertidal mudflats (Admiraal 1984; Underwood 1994), produce EPS when migrating through the sediment (Edgar & Pickett-Heaps 1984) in response to light and tidal conditions (Hay et al. 1993). This pattern of migration allows the cells to position themselves at the sediment surface during the daylight exposure period, and retreat into the sediment matrix before the return of the tide or when dusk falls (Round 1981). However, one must bear in mind that Staats et al. (2000a, b) showed that the amount of secreted polysaccharides is far in excess of the theoretical needs for locomotion only. These workers hypothesized that it is mainly the result of the overflow metabolism, presumably due to nutrient limitation. The EPS binds sediment particles together at the mud surface and are the key factor in the process of biostabilization of sediments (Paterson 1994; Underwood & Paterson 1993a, 1993b). De Jonge (1985) showed that such a biostabilization effect is also governed by physics since

physical processes are responsible for diatom assemblage type ("epipelon-like" vs. "epipsammic-like").

EPS is often a constant fraction (~25%, Underwood et al. 1995) of the total water-extractable ('loosely attached') carbohydrates pool which has been shown to be linearly related to the chlorophyll *a* content of the sediment (Underwood & Paterson 1993a, b; Underwood & Smith 1998b). This functional linear relationship between both variables can be interpreted as the result of: (i) water-extractable carbohydrates are predominantly produced by epipelic diatoms, and (ii) the turnover rate of carbohydrates is relatively high (thus allowing a rather constant carbohydrate : chlorophyll *a* ratio). In addition, this simple linear relationship has been shown to be valid for a number of estuarine intertidal mudflats with a predominant community of epipelic diatoms (Underwood & Smith 1998b). If so, the linear relationship between water-extractable carbohydrates and chlorophyll *a* could be used as a tool to predict the potential biostabilization effect due to microphytobenthos, and as an important biological criterion for mudflat classification (see Dyer et al. 2000) despite the strong seasonal variation in biomass of diatoms (de Jonge & Colijn 1994). The value of this model is in its simplicity and apparent general applicability.

In this study, we propose to use the simple empirical relationship to further analyze the effect of the potential influence of microphytobenthos on sediment biostabilization, by investigating the interaction between physical and biological processes that occur in mudflats. Intertidal mudflats exhibit a number of geomorphological structures with distinct physical characteristics that are likely to affect biological processes, including the relationship between water-extractable carbohydrates and chlorophyll *a*. Therefore, we have studied the spatial variations of both the contents of chlorophyll *a* and water-extractable carbohydrates across a macro-tidal mudflat at four different sites in the Humber estuary, UK. The functional linear relationship between both variables was quantified as a function of depth and compared to the sedimentary structures present along a cross-shore transect.

Material and methods

Study area

Sampling was carried out on July 6, 1997 at Skeffling in the Humber estuary, UK. The study area and the environmental conditions are described in detail by Christie et al. (2000). Four sites (designated A, B, C and D) were chosen along a cross-shore transect, with site A closest to the shore at the boundary between the narrow saltmarsh and the mudflat, and site D at the seaward end approximately in the middle of the mudflat. Fig. 7.1 shows an aerial view

of the cross-shore transect, indicating the location of the sampling sites. The inserted photographs show the morphological features of the sediment surface at each of the four sites.

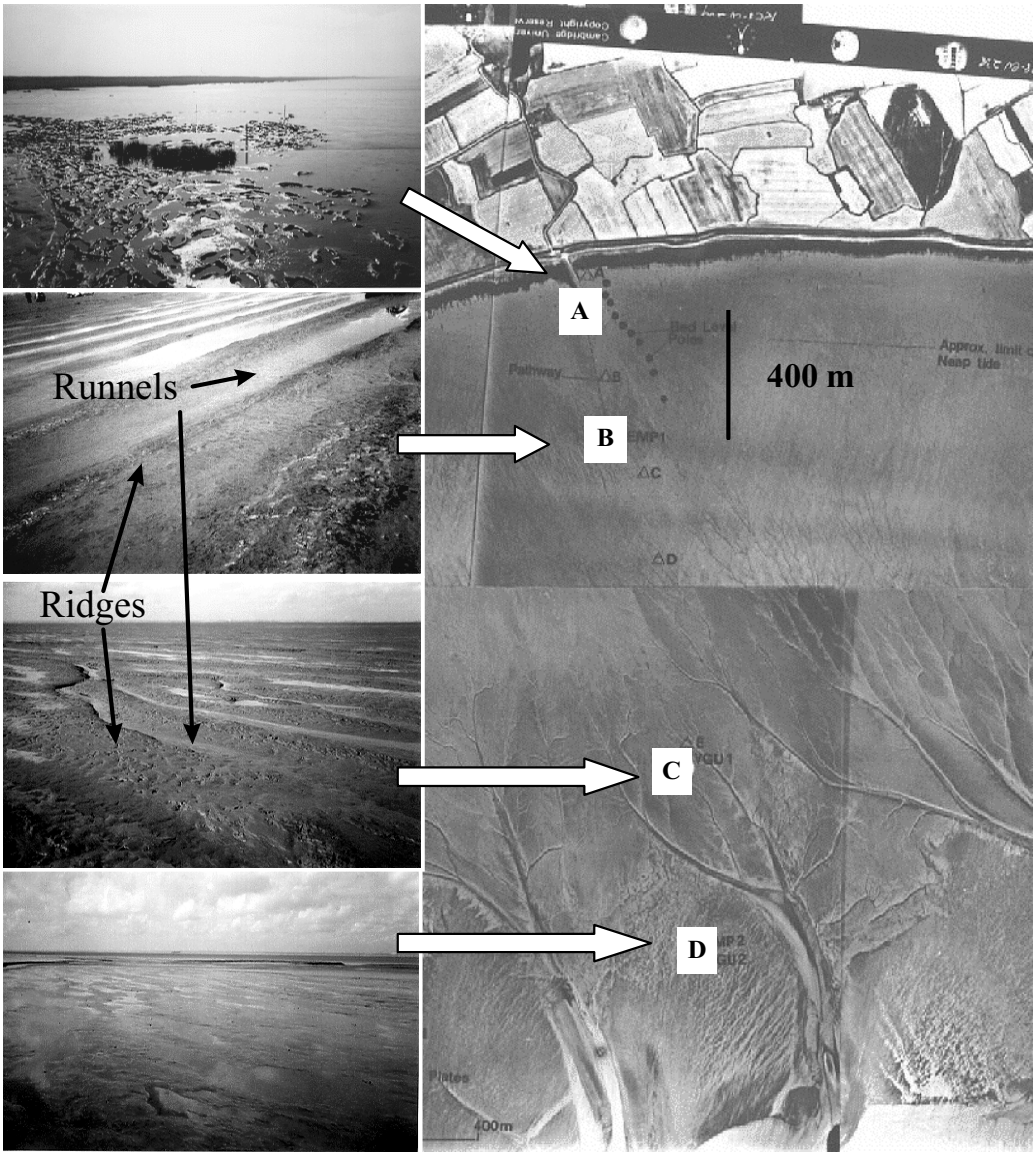

Figure 7.1. Aerial view of the sampling area at Skeffling in the Humber estuary. The cross-shore transect and the four sampling sites (A, B, C and D) are presented. The transect was about 1600 m long. Sites A and B were located in the upper part of the mudflat, while C and D were in the middle of the flat where major channels are present. The series of 4 photographs shows the surface of the sediment at the different sites: at site A the sediment surface was flat with overlying fluid mud (tracks made during sampling are also apparent), there was a ridge and runnel system at sites B and C, site D was more sandy with no real ridge and runnel system. Ridges are the emergent crests, and runnels are troughs containing sea water.

sites A, B and C were muddy while site D contained more sand. Site A was characterized by a flat surface covered by fluid mud. Sites B and C possessed a well-developed shore-normal ridge and runnel system. Ridges are crests, which emerge at low tide and tend to dry out during emersion. Runnels are troughs - drainage-like structures -, which contain slow-running water or stagnant water during most of the period of emersion (Fig. 7.1: photographs of sites B and C). This ridge and runnel system covered most of the cross-shore transect and it was therefore a prominent feature of the mudflat at Skeffing. Many mudflats from other estuaries possess a similar morphology of ridges and runnels. Site D was different because it did not have a ridge and runnel system, but crests and troughs were nevertheless apparent. Detailed information about the physical properties of the sediment at all sites as well as their spatial-temporal dynamics and the morphology is provided in companion papers (Christie et al. 2000; Whitehouse et al. 2000).

Sampling strategy

Cut-off 50-ml syringes were used to sample the sediment. Two layers of sediment were retained for analysis: the surface (0-5 mm) and the sub-surface (5-10 mm). Samples were taken along 2 short long-shore transects (2 m apart, each about 3-4 m long), each covering 3 successive ridges and runnels. A total of 6 cores on ridges and 6 cores in runnels were collected at each site. The total number of samples was 96 (4 sites, 12 cores and 2 depth layers). Each sample was mixed in the field immediately and divided into 3 sub-samples. One of these sub-samples was used for biological measurements (this study) while the other two were used for measurement of physical characteristics (Christie et al. 2000).

Chlorophyll a measurements

Sub-samples devoted to chlorophyll *a* measurement were freeze-dried and kept in the dark at -80°C until further processing. The chlorophyll *a* content of the dried sediment was extracted in 90% acetone during 18 h in the dark with agitation; chlorophyll *a* was then measured using the fluorometric method, and data were transformed into concentration units expressed as mg Chl $a \cdot m^{-2}$.

Water-extractable carbohydrate measurements

The water-extractable carbohydrate content of the sediment was quantified using a spectrophotometric assay (Dubois et al. 1956). Sediment carbohydrates were operationally separated into water-extractable and bulk carbohydrate fractions (Underwood et al. 1995). Sediment samples, taken from the field were frozen and then freeze dried. The water-

extractable carbohydrate portion of the sample was then extracted from the sediment using saline solution. The water and sediment were mixed to form a slurry which was incubated at 20°C for 15 min. The liquid phase containing water-extractable carbohydrates was then separated by centrifugation (1500×g, 15 min). 0.5 ml of 5 % w/v of phenol was added to 1 ml of the supernatent. 2.5 ml of concentrated sulfuric acid (H_2SO_4) was added to the mixture using a pump dispenser. The mixture was incubated for 60 min and sugar concentration determined against glucose standards (Liu et al. 1973).

Relationship between Chlorophyll a and water-extractable carbohydrates

The relationship was quantified using a simple linear regression analysis. A model I regression was used since a functional relationship between water-extractable carbohydrates, as the dependent variable, and chlorophyll *a*, as the independent variable, was assumed. When necessary, slopes of the regression lines were compared using a t-test (for 2 slopes) or a covariance analysis (for more than 2 slopes), according to the procedures reported in Zar (1984).

Results

Analysis of the relationship between water-extractable carbohydrates and Chlorophyll a

When all data (from the different sites, depth horizons and structures) were pooled, a statistically significant positive relationship was found between water-extractable carbohydrates and chlorophyll *a* (Fig. 7.2, Table 7.1). However, the coefficient of determination was low. The linear model explained only 40% of the observed variability. Hence, when the origin of the samples was not taken into account, it appeared that chlorophyll *a* was a weak predictor of the content of water-extractable carbohydrates, and consequently of the potential biostabilization. Therefore, the model was applied with respect to the different origins of the samples that were likely to affect the relationship. When the 2 sediment depth layers (surface 0-5 mm, and sub-surface 5-10 mm) were analyzed separately, significant positive relationships were found for both (Fig. 7.3, Table 7.1). However, the slope for the surface sediment was significantly higher than that for the sub-surface sediment ($P<0.05$). The coefficient of determination was also much higher for the surface layer (62%) compared to the sub-surface (19%).

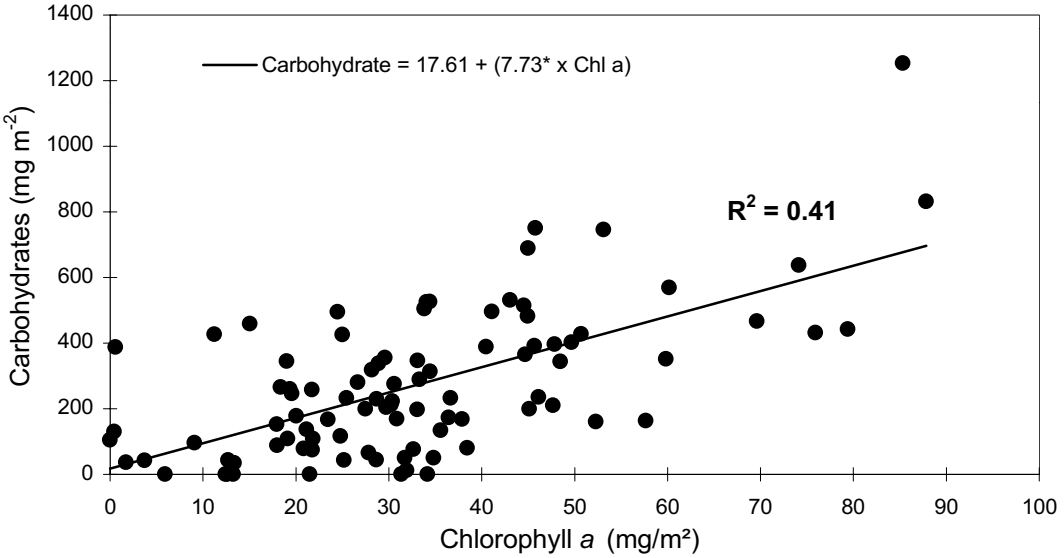

Figure 7.2. All data are pooled together (from the different sites, from surface and sub-surface sediment, from ridges and runnels). There is a significant linear relationship between water-extractable carbohydrates and chlorophyll *a*. The slope is significantly different from 0 (P<0.001), but the intercept is not (P>0.05).

Table 7.1. Statistical results of the different regression lines between carbohydrate (Y) and chlorophyll *a* (X) concentration (mg m^{-2}). P is the probability of a Type I error.

Type of relationship	Regression equation	Intercept	Slope
All stations, all depths	Y = 17.60 + 7.73 X	$P > 0.05$	$P < 0.001$
Station A only, all depths	Y = 137.03 + 5.38 X	$P > 0.05$	$P \leq 0.05$
Station B only, all depths	Y = 64.94 + 6.57 X	$P > 0.05$	$P \leq 0.01$
Station C only, all depths	Y = -130.44 +10.87 X	$P > 0.05$	$P < 0.001$
Station D only, all depths	Y = 130.47 - 1.00 X	$P < 0.05$	$P > 0.05$
Surface sediment only (0-0.5 cm), all stations	Y = -95.57 + 9.99 X	$P > 0.05$	$P < 0.001$
Sub-surface sediment only (0.5-1 cm), all stations	Y = 124.79 + 5.13 X	$P < 0.05$	$P < 0.01$
Ridges at stations B and C, all depths	Y = 25.50 + 8.81 X	$P > 0.05$	$P < 0.001$
Runnels at stations B and C, all depths	Y = 222.37 - 1.50 X	$P < 0.05$	$P > 0.05$

Site effect on the relationship

The results (Fig. 7.4, Table 7.1) showed that the relationship between chlorophyll *a* and water-extractable carbohydrate, measured at all depths, was statistically significant for sites A, B and C, and that there was no significant difference found between the slopes of the regression found at each of these sites (analysis of covariance, $P>0.05$). Site D was different in that there was no significant relationship between these variables ($P>0.05$).

Effect of topographic features

To examine the influence of topographic variation on the relationship between water-extractable carbohydrates and chlorophyll *a*, data was sorted into samples from both depths collected from the ridge and runnel features at sites B and C (Fig. 7.5, Table 7.1). Results clearly indicate an effect of these geomorphological structures on the distribution of EPS and consequent potential biostabilization. There was no significant relationship between carbohydrates and chlorophyll *a* for the sediment sampled in runnels ($P>0.05$); both variables were independent. On the other hand, there was a highly significant positive relationship for the sediment sampled on top of ridges ($P<0.001$). It is also apparent that chlorophyll *a*

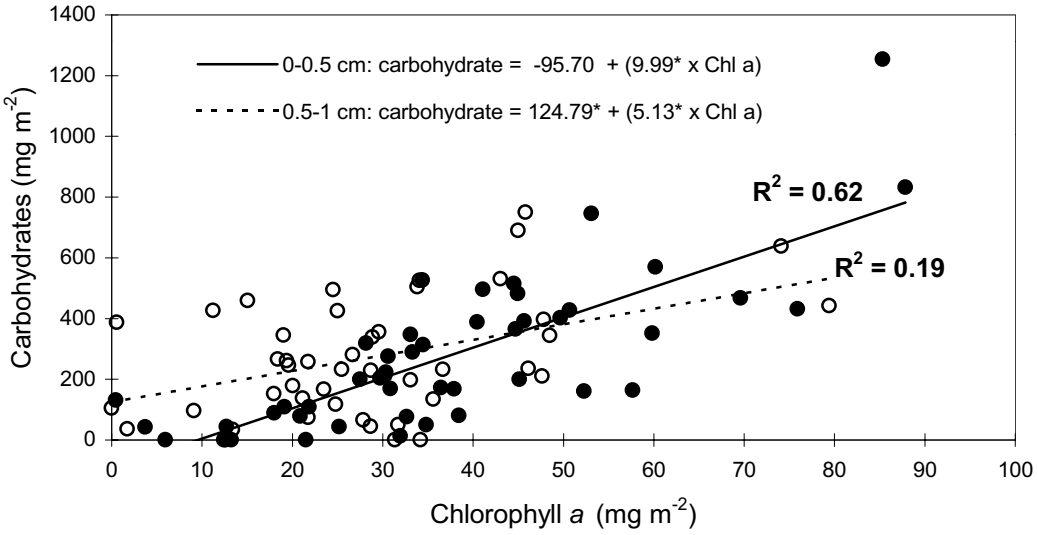

Figure 7.3. Comparison of the relationship between water-extractable carbohydrate and chlorophyll *a* in surface sediment (0-5 mm, ●) and sub-surface sediment (5-10 mm, o). Both slopes are significantly different from 0 ($P<0.001$ and $P<0.01$ for surface and subsurface sediment, respectively); the intercept is not different from 0 for the surface sediment ($P>0.05$), but is different from 0 for the sub-surface sediment ($P<0.05$).

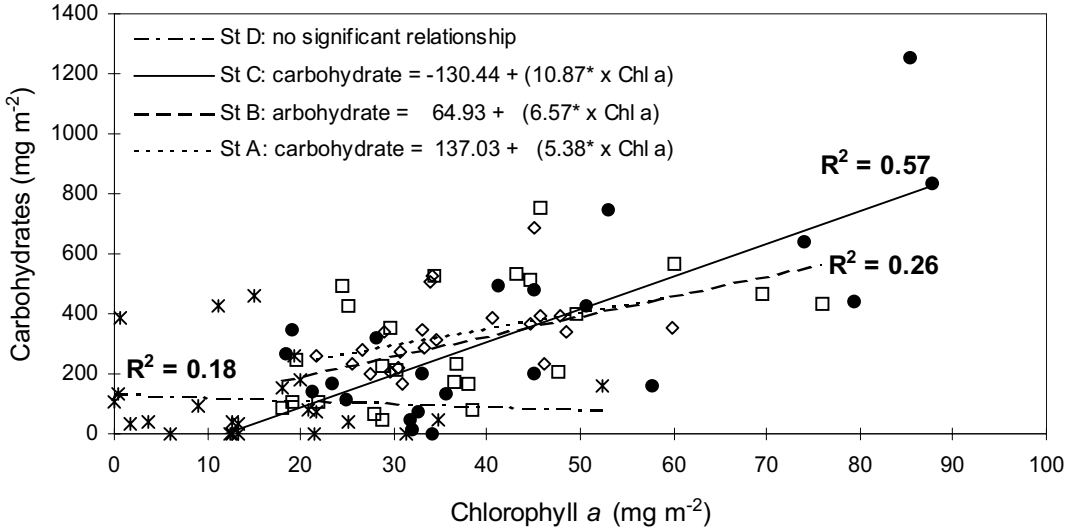

Figure 7.4. Comparison of the relationship between water-extractable carbohydrates and chlorophyll *a* at station A (●), station B (□), station C (◊) and station D (∗). The slope of the regression line is significantly different from 0 at sites A, B and C; intercepts are not different from 0. There was no significant relationship at site D. Site A: Water-extractable carbohydrates = 137.03 + 5.38* chl *a*, with R^2 = 0.18; Site B: Water-extractable carbohydrates = 64.93 + 6.57* chl *a*, with R^2 = 0.26; Site C,: Water-extractable carbohydrates = -130.44 + 10.87* chl *a*, with R^2 = 0.57. Slopes at sites A, B and C are not statistically different (Analysis of Covariance, P>0.05).

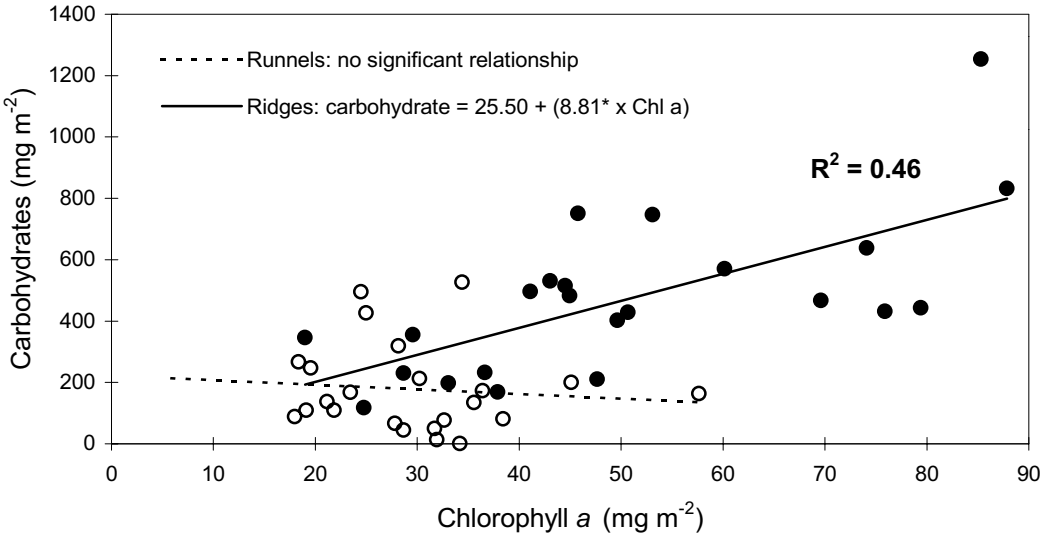

Figure 7.5. Comparison of the relationship between water-extractable carbohydrates and chlorophyll *a* between ridges (●) and runnels (○). There is no significant relationship in runnels. On ridges, the slope is significantly different from 0 (P<0.001); the intercept is not different from 0 (P>0.05).

concentrations in runnels were lower than on ridges; the highest concentration recorded in runnels was much lower than that measured on ridges. Similarly, the average water-extractable carbohydrates concentration in runnels (around 200 mg m^{-2}) corresponded to the lowest levels measured on ridges (the highest levels being 4- or 5-fold higher).

Discussion

The site

The physical and biological characteristics of this site on the Humber have been well-documented (Black and Paterson 1998). The sedimentological parameters were investigated in various studies (Black et al. 1998; Dyer et al. 2000). As part of this earlier work the stability of the site was measured by the *in situ* SEA CAROUSEL flume (Amos et al. 1998). The latter study showed that there was considerable variation in stability between stations on a similar transect and the authors interpreted this variability as a response to both physical and biological factors. The highest stability was found at site C where high levels of chlorophyll *a* were also found. This work suggested that more information on the spatial distribution of chlorophyll *a* and the mechanistic link between chlorophyll *a* and sediment erodibility was worthy of further investigation. Therefore, the Underwood & Smith (1998b) model, if validated, should provide a rapid and easy assessment of potential biostabilization if it is shown that the relationship hold widely and that natural spatial heterogeneity is not sufficient to prevent the generic use of the formulation.

The Underwood & Smith model

The relationship between chlorophyll *a* and water-extractable carbohydrates is thought to stem from the locomotion by epipelic diatoms, which form the major component of the majority of microphytobenthic assemblages. However, it may be hypothesized that the relationship is also due to overflow metabolism (Staats et al. 2000a, b; chapter 3, 5). Epipelic diatom locomotion is through the secretion of mucopolysaccharide material through the raphe slit (Edgar & Pickett-Heaps 1984). This material can be measured via the simple assay outlined (methods) but the actual extent of polymerization and the molecular make–up of the polymers is still unclear (Taylor et al. 1999). During the extraction procedure, an operational separation takes place which fractions the material produced by diatoms along with components of other organic material in the sediment. This has led to a variety of terminology in the literature (Decho 1990; Paterson 1990; Sutherland et al. 1998), despite the attempts of

Underwood et al. (1995) to provide a common framework. Water-extractable carbohydrates, as used here, are the operational fraction that is found in the supernatant after saline or water extraction from the sediment. This material contains both polymeric (water-extractable EPS) and short chain sugars that will be detected by the Dubois assay. The strong relationship between fractions of this material and diatom biomass (as chlorophyll *a*) is therefore quite remarkable and suggests that there is a strong coupling between the material produced by diatoms and the material recovered by the operational procedure.

However, water-extractable carbohydrates are readily dissolved in water or may be degraded (van Duyl et al. 1999). Any relationship between them and microphytobenthos biomass will therefore be dynamic. The importance of these carbohydrates for biostabilization of the sediment may therefore be limited. Carbohydrates that do not easily dissolve in the water and are more recalcitrant to decomposition may accumulate in the sediment. This carbohydrate fraction is therefore not usually correlated to microphytobenthic biomass by a simple linear relationship (De Winder et al. 1999). Staats et al. (1999) have shown that the epipelic diatoms *Cylindrotheca closterium* and *Navicula salinarum* produced exopolysaccharides that were attached to the cells and contained negatively charged groups such as uronic acids and sulfated sugars and they suggested that these carbohydrates may be more important for sediment stabilization.

Results of the present study indicate that the vertical distribution of the water-extractable material and related chlorophyll *a* is important in the strength of the relationship between the two. This confirms the microspatial examination of chlorophyll *a* and carbohydrate distribution carried out by Taylor & Paterson (1998). The stronger relationship between chlorophyll *a* and water-extractable carbohydrates at the surface might be explained by the fact that epipelic algae are more active at the surface, both in terms of motility and photosynthesis; consequently, they produce more carbohydrates per unit of biomass. This conflicts with the report of Underwood & Smith (1998a), who found that EPS production occurred also in the dark. However, De Winder et al. (1999) showed that during emersion of an intertidal flat during the day, water-extractable carbohydrates showed a dramatic increase, particularly in the top few mm (see also chapter 5). They also showed that this increase was light dependent. Staats et al. (2000a) showed that EPS production in a diatom mat as well as in an axenic culture of the benthic diatom *Cylindrotheca closterium* was strictly dependent on photosynthesis. Therefore, the strength of the relationship between carbohydrates and chlorophyll *a* as well as the ability to predict biostabilization due to microphytobenthos would most likely be improved considerably by decreasing the depth interval of sampling at the surface (i.e., taking 1 mm, or even less, instead of 5). By decreasing the depth interval of sampling over the top few mm of the sediment an even more active part of the diatom community is selected and this increases the specific carbohydrate content enormously (Taylor & Paterson 1998).

Inter-site variability

By analyzing the data separately for each sampling site, it is possible to point out the effect of sediment characteristics and, to a lesser extent, the effect of the duration of the emersion period. There was thus a contrast between the muddy sites and the more sandy one, which suggests that this relationship only holds for muddy sediments. The higher proportion of sand at site D (more than 50%, compared to 4%, 11-15%, and 19-38%, respectively, at sites A, B and C; see table 1 in Christie et al. 2000) likely prevents biostabilization from occurring. We hypothesize that this may be due to the fact that, at this particular site, the benthic community was very likely mainly composed of epipsammic diatoms, which secrete little polysaccharides, while it was dominated by epipelic diatoms at the other 3 sites. This would be in complete agreement with the observations of Underwood & Smith (1998b), and is consistent with the conclusions of de Jonge (1985) according to which physical processes control diatom community type, hence, biostabilization. This further indicates that the relationship is not necessarily valid for an entire mudflat. The coefficients of determination (Fig. 7.4) also show that the model was the most reliable at site C where the ridge and runnel system was the most developed. Interestingly, this was also the site selected by Amos et al. (1998) as the area of greatest biology impact on sediment stability.

Intra-site variability

Several possible mechanisms could explain the difference between geomorphological structures. The main difference between ridges and runnels comes from their physical characteristics. Ridges are completely emerged during low tide and progressively dry out, while runnels are covered with slow-running seawater and never dry out (Fig. 7.1). Therefore, in the case of ridges, conditions at the surface sediment (consolidating mud, no excess of water) probably allow a short-term accumulation of newly produced carbohydrates by the upwards migrating epipelic microalgae. However, there must also be a rapid turnover of this carbohydrate pool in order to be consistent with the observed linear positive relationship between these carbohydrates and the microalgal biomass.

In the runnels, the newly produced carbohydrates are probably quickly dissolved in the overlying water and may have been washed out with the slow-running water. This would explain why the runnels are apparently not capable of accumulation of carbohydrates and why the contents of both carbohydrates and chlorophyll *a* are lower than those measured on top of the ridges. Alternatively, it is also possible that the overlying water in the runnels would have prevented the epipelic microalgae from migrating to the surface or that the turbid water would have limited photosynthesis and consequently the production of carbohydrates.

In summary, it appears that the physical characteristics prevailing in runnels prevent carbohydrate-mediated stabilization of the sediment and probably caused it to be self-

sustaining. The opposite may be true for the ridges. A comparable observation has been reported by Staats et al. (2001). These authors found that epipelic diatoms could only colonize that part of the mudflat where the median grain size was sufficiently small (see also, de Jonge et al. 1985) and the emersion period sufficiently long at low tide. However, once established, the diatom community increased its own suitable environment by binding and consolidating mud that was deposited. Van de Koppel et al. (in press) give a theoretical basis for this and present experimental evidence for the possibility of two stable states on intertidal mudflats: low grain-size with diatom mats and large grain-size without diatoms. The system could move from one state to the other for instance by physical forcing.

Conclusions

This potential biostabilization effect, as inferred from the carbohydrate - chlorophyll *a* relationships, has to be interpreted in the physical context of mud consolidation during emersion. Due to purely physical processes (dewatering, compaction), the sediment drying out on ridges (and more generally on any exposed structures) during low tide is likely to increase its stability. However, information from previous work has shown that the intuitive idea that water content declines and drying out occurs during the exposure period is not always correct. The water content of exposed sediment is often fairly constant (George 1994), and this can also be reflected in wet bulk density measurements. In addition, stability can be decoupled from water content as in instances where low bulk density systems are nevertheless stable, often due to biofilm formation. Similar results have been produced for the current site which show that although the bulk density of site C was low (see Christie et al. 2000), the stability was relatively high (Amos et al. 1998). This information is likely to apply to the ridge systems.

Nevertheless, drying does sometimes occur as the sediment is exposed and, although the time series is not always predictable, the influence of drying will impact on sediment stability. This may have a synergistic effect and actually promote biostabilization, which in turn, enhances sediment stability. In runnels, there may be transient deposition of sediment, which does not consolidate because of the presence of water. The latter also seems to prevent biostabilization from occurring. As a result, there seems to be a positive feedback between physical and biological processes (biostabilization exacerbates sedimentological characteristics) on both ridges and runnels, which leads to an opposite sedimentary behaviour of two co-occurring structures.

CHAPTER 8

DISTRIBUTION OF EXTRACELLULAR CARBOHYDRATES IN THREE INTERTIDAL MUDFLATS IN WEST-EUROPE

J.F.C. de Brouwer, E.M.G.T. de Deckere & L.J. Stal

Abstract

In this study the spatial distribution of two operationally defined extracellular carbohydrate fractions (water- and EDTA-extractable carbohydrates) were examined in three intertidal mudflats in West Europe (Dollard, the Netherlands; Marennes, France; Humber, UK). The three mudflats were sampled along cross-shore transects and sediment cores were sliced to a depth of 5 cm. Carbohydrate contents showed little variation with depth but contents varied between the stations along the transect reflecting the variation within the different mudflats. Carbohydrate contents were also significantly different between mudflats and the carbohydrate contents of the stations within a mudflat grouped together resulting in separate clusters for the different mudflats. Similar results were obtained when the Marennes-Oléron mudflat was investigated on a temporal scale indicating that dynamics in extracellular carbohydrate contents occurred over larger areas, typically the size of mudflats. In the surface 0.5 cm of the sediment, water-extractable carbohydrates showed a correlation with both chlorophyll *a* content and median grain size while EDTA-extractable carbohydrates were only correlated with median grain size. Incubation experiments also showed the importance of microphytobenthos as a source of extracellular carbohydrate especially when subjected to the light. Analyses of the monosacchride distribution of the carbohydrate fractions revealed that the carbohydrate composition was largely similar between the areas investigated. Structurally the carbohydrates that were found in these sediments seemed to represent a biorefractory part of the carbohydrates that remained after rapid degradation of the labile component of the freshly produced extracellular sugars.

Introduction

Intertidal mudflats are dynamic and biologically highly productive areas. The dominant primary producers in these environments are benthic epipelic diatoms (Bacillariophyceae*)* (Admiraal 1984; Underwood & Kromkamp 1999). These organisms are considered to be an important source of carbohydrates because a substantial part of the fixed carbon is excreted into the surrounding in the form of extracellular sugars (Goto et al. 1999; Smith & Underwood 2000). Under conditions that favour diatom growth this may result in the formation of diatom mats. These biological structures enhance the stability of the surface sediment layer (Paterson 1997; Amos et al. 1998; Kornman & de Deckere 1998) and cause net deposition of fine-grained sediments (Frostick & McCave 1979; Paterson et al. 2000; chapter 6). Several studies have therefore focused on the relationship between chlorophyll *a* and extracellular carbohydrates in intertidal mudflats. Underwood & Smith (1998b) proposed a simple relationship between the two parameters that was valid for a range of intertidal mudflats in the UK. Meanwhile, other studies have shown that the predictive power of this relationship is increased when different geomorphological features are taken into account (chapter 7) and when the vertical resolution of the slicing method is increased (Kelly et al. 2001).

Apart from the dependence between chlorophyll *a* and extracellular carbohydrate, sediment grain size was also correlated with carbohydrate content in the sediment (Paterson et al. 2000; van de Koppel et al. 2001; chapter 6). This has been explained in several ways. Paterson et al. (2000) suggested that this was due to the fact that diatoms growth rates are higher in muddy sediments (see also van de Koppel et al. (2001)) and because diatom might trap fine grained sediment particles. Staats et al. (2001) added that this correlation was possibly a result of the hydrodynamic regime that has a similar effect on microphytobenthos and mud particles (see also Christie et al. (1999)).

The spatial distribution of extracellular carbohydrates has been investigated at different scales (Taylor & Paterson 1998; Underwood & Smith 1998b; de Winder et al. 1999; Paterson et al. 2000; Staats et al. 2001; chapter 7). All these studies presented a quantitative description of extracellular carbohydrates present in intertidal sediments. Information regarding the composition of these carbohydrates may provide information about the source and status of the carbohydrates present (Cowie & Hedges 1984). Although some knowledge is available about carbohydrate composition in intertidal mudflats (Taylor et al. 1999; chapter 5) to the authors knowledge no information exists at present that describes spatial patterns in the composition of extracellular carbohydrates in intertidal mudflats.

The aim of this study was to investigate the spatial distribution of extracellular carbohydrates in intertidal sediments both in a quantitative and qualitative way. Three intertidal mudflats that were situated in different geographical areas in Northwest Europe were investigated to identify whether spatial patterns in carbohydrate distribution differed depending on the area that was studied. The intertidal mudflats were sampled over cross-

shore transects and to a depth of 5 cm. In this way spatial heterogeneity was determined with depth, within intertidal mudflats and between intertidal mudflats. The distribution of extracellular carbohydrate was analyzed quantitatively by measuring carbohydrate contents in the sediment and qualitatively by analyzing the monosaccharide distributions of these carbohydrates. In order to explain the observed patterns in the extracellular carbohydrate distribution the relationships with grain size and chlorophyll *a* were examined. Furthermore, production of extracellular carbohydrate by microphytobenthos was examined and compared to the contents of carbohydrates found in the field. These results are discussed in relation to processes that determine the dynamics of extracellular carbohydrates on intertidal mudflats.

Materials and methods

Study areas

Samples were collected from three estuaries, The Bay of Marennes-Oléron, France, The Humber estuary, UK and the Ems-Dollard estuary, the Netherlands. In the Marennes-Oléron Bay a 2500 m long cross-shore transect was sampled at the Montportail-Brouage mudflat (for details see, Galois et al. 2000). The Skeffling mudflat, which is situated at the northeastern part of the Humber estuary was sampled over a 4 km cross-shore transect (Black & Paterson 1998). In the Dollard (which is the upper reach of the Ems-Dollard estuary) 3 stations were sampled along the main channel over a distance of approximately 4 km. The Marennes and the Humber mudflat are subject to a macrotidal regime while the Dollard mudflat is mesotidal. Average emersion times for the sampling stations are shown in Table 8.1. For the Marennes and the Humber mudflat, emersion times were highest closest to the shore and decreased in seaward direction. This was also the case in the Dollard mudflat, however, differences in emersion times between the different stations were far less pronounced. The mudflat slopes were comparable for three investigated areas and varied between 1:1000 and 1:1100. Mudflat morphology in the Dollard mudflat was mostly planar with dewatering structures being present at the lower parts of the mudflat. In contrast, large areas of the Marennes and the Humber mudflat were characterized by extended ridge-runnel features (Dyer 1998). These are elongated parallel corrugations with a width of 0.5-1.5 m and height of 20-40 cm. These features are generally oriented in a shore normal position. During low tide the ridges are exposed while water is regularly trapped in the runnels. A detailed description of morphological characteristics and hydrodynamic regime in the three intertidal areas was given by Whitehouse et al. (2000).

Table 8.1. Average emersion time, median grain size (top 0.5 cm), chlorophyll *a* content (top 0.5 cm) in the Marennes mudflat, the Humber mudflat and the Dollard mudflat.

Site	Average emersion time (hh:mm)	Median grain size (μm)	Chlorophyll *a* ($\mu g \cdot g^{-1}$)	Visible diatom patches
Marennes, April 1997				
Station 1	08:50	13.4	8.8	yes
Station 3	01:50	16.3	9.6	yes
Station 4	00:45	14.5	3.0	no
Humber, July 1997				
Station A	10:00	12.5	7.2	yes
Station B_1	08:40	13.5	3.9	no
Station B_2		13.3	3.2	no
Station C_1	06:50	24.9	5.7	no
Station C_2		22.4	4.5	no
Station D_1	05:40	38.4	1.4	no
Station D_2		49.7	1.0	no
Dollard, July 1997				
Station 1	04:50	26.0	4.4	no
Station 2	04:35	59.4	4.8	no
Station 3	04:00	91	10.3	yes

Sampling

Sediment samples were collected from the Marennes mudflat in November 1996, April 1997 and November 1997. The Humber and the Dollard mudflat were sampled in July 1997. In the Humber mudflat samples were collected from both the ridges and the runnels (samples taken for the ridges are denoted by the station name followed by a 1, while runnels are denoted by station name followed by a 2). In contrast, the Marennes mudflat was sampled on the ridges exclusively. Sampling (and subsequent analysis) was done in triplicate except for the Humber mudflat, where 5 replicate cores were taken for pigment and carbohydrate analysis. Samples for carbohydrate and pigment analyses were collected using stainless steel cores (∅: 12 mm). The cores were sliced at depth intervals of 0.5 cm to a depth of 1 cm and at 1 cm intervals to a depth of 5 cm. Samples for grain size analyses were collected using perspex cores (∅: 30 mm) and sliced at 0-0.5 cm. Slicing took place at the field site and samples were directly stored in

the dark at 0°C. Back in the laboratory samples were immediately frozen at –20 °C and freeze dried prior to analyses.

Carbohydrate analysis

Carbohydrate contents were determined using procedures in chapter 6. In short, two operational fractions were analyzed. The water-extractable carbohydrate fraction was obtained by extraction of the lyophilized sediment with distilled water (1h, 30°C). After centrifugation of the suspension at 6000×g, EDTA-extractable carbohydrates were obtained by extraction of the sediment pellet with 0.1 M Na_2EDTA (16 h, 20°C). Carbohydrates were quantified using the phenol-sulfuric acid spectrophotometric assay (Dubois et al. 1956).

Pigment analysis

Freeze dried sediment samples were ground and sonicated in 90:10 methanol : NH_4Ac for 1 h. Subsequently, the extracts were centrifuged at 1000 g for 5 min. The pigments were analyzed by HPLC as described by Barranguet et al. (1997).

Monosaccharide analysis

Samples taken during the campaigns in the Marennes mudflat (April 1997), the Humber mudflat (July 1997) and the Dollard mudflat (July 1997) were analyzed for their monosaccharide distributions. The procedures to analyze the monosaccharide distribution in the water- and EDTA-extractable carbohydrate fractions are described in chapter 5. In short, the extracts were filtered using ultrafiltration centrifuge tubes with a cut off of 1 kD (Amicon) in order to remove salts and small sugar moieties. Subsequently, the polymers were hydrolyzed and methanolyzed to their methylglycosides using 0.5 ml of 2 M HCl in methanol for 16 h at 80 °C. Finally, the methylglycosides were hydrolyzed to monosaccharides using 1 ml of 2 M trifluoro-acetic acid (1 h at 120 °C). The monosaccharide distribution was analyzed by HPLC equipped with a Pulse Amperometric Detector (PAD).

Grain size analysis

Median grain size, mud- and silt content were determined on lyophilized sediment samples using a Malvern Particle Sizer operated with a 300 mm lens.

Incubation experiment

To examine the effect of light and dark conditions on the one hand and the effect of exposure and immersion of the sediment on the other hand an incubation experiment was carried out in April 1997. For this purpose 12 sediment cores were taken from the Marennes mudflat (station 4) and subjected to 4 different treatments (n=3) in the laboratory. The cores were equally divided between two aquariums that were bubbled with air. In each aquarium half of the cores were situated just above the water surface while the other cores were situated just under the water surface (≈ 1 cm). One aquarium was placed in the light (incident irradiance: 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) while the other aquarium was placed in the dark. The cores were incubated for a period of 48 h at 20 °C. At the end of the incubation, water was carefully removed from the immersed cores, subcores (n=2) were taken from each core, sliced as described before and analyzed for carbohydrate and pigment contents.

Statistical analysis

A nested ANOVA design was used to test the effects of the campaigns, the stations along transects, the cores taken at a station and the various depth intervals within a core on the carbohydrate contents. Stations along transects were nested in the campaigns and cores taken at a station were nested in station which were nested in the campaigns. Campaigns, stations and cores were treated as random factors whereas depth intervals were treated as a fixed factor.

For the incubation experiments a comparable design was used. Treatment and depth were treated as fixed factors while cores and subcores were treated as random factors. The subcores were nested in the original cores. All statistical analyses were performed on log-transformed data to ensure homoscedasticity of the dataset. Data were analyzed using the Statistica 5.5 package.

Results

Carbohydrates

In Fig. 8.1 typical vertical carbohydrate profiles are shown for the studied mudflats. These profiles are a good representation of the vertical patterns in extracellular carbohydrates that were found. Carbohydrate profiles were either constant or increased slightly with depth (for example Fig. 8.1B, EDTA-extractable carbohydrates). Furthermore, EDTA-extractable

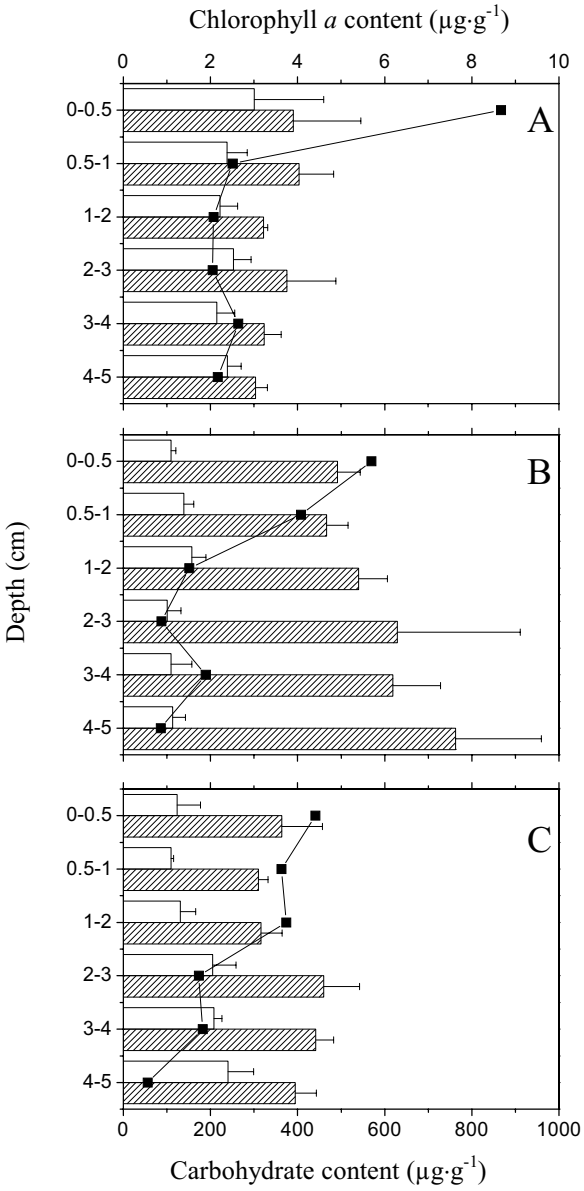

Figure 8.1. Vertical profiles (mean ± 1 SD) of water- and EDTA-extractable carbohydrate contents (black and white bars, respectively) and chlorophyll *a* contents (■) in the Marennes mudflat, station 1, April 1997 (A), Humber mudflat, station C_1, July 1997 (B) and the Dollard mudflat, station 1, July 1997.

carbohydrate contents were always higher compared to the contents of water-extractable carbohydrate. The complete set of vertical carbohydrate profiles that was measured over the three investigated mudflats was subjected to statistical analyses. From this a number of general observations were done. Water-extractable carbohydrate contents were constant with depth, and varied between 0-300 $\mu g \cdot g^{-1}$. EDTA-extractable carbohydrates increased slightly but significantly with depth ($F_{(5, 253)}=6.7$; $p<0.001$) and contents varied between 200-1200 $\mu g \cdot g^{-1}$. Both water- and EDTA-extractable carbohydrates differed significantly between the mudflats ($F_{(4, 343)}=5.6$; $p<0.01$ and $F_{(4, 343)}=12.7$; $p<0.001$ for water- and EDTA-extractable

carbohydrates, respectively) and between the stations in the mudflats ($F_{(14, 343)}$ =33.6; p<0.001 and $F_{(14, 343)}$=26.5; p<0.001, for water- and EDTA-extractable carbohydrates, respectively). When comparing water- and EDTA-extractable carbohydrates it was observed that the content of EDTA-extractable carbohydrate was higher than water-extractable carbohydrate. Furthermore, in the presence of diatom biofilms carbohydrate contents were not elevated in the upper 0.5 cm relative to the deeper sediment layers.

Because carbohydrate contents were constant with depth for the water-extractable carbohydrates and varied only slightly for the EDTA-extractable carbohydrates, contents of both fractions were averaged over depth. When the EDTA-extractable carbohydrates were plotted against water extractable carbohydrates, the stations within mudflats appeared to cluster together (Fig. 8.2A). The stations that did not fit into these clusters, station A in the Humber and station 1 in the Dollard, had clearly different properties that accounted for this non-clustering. Station A in the Humber was situated very close to the salt march and to a fresh water inlet. The sediment at this station was homogeneous with a high water content (Christie et al. 2000), and differed morphologically from the other stations along the transect

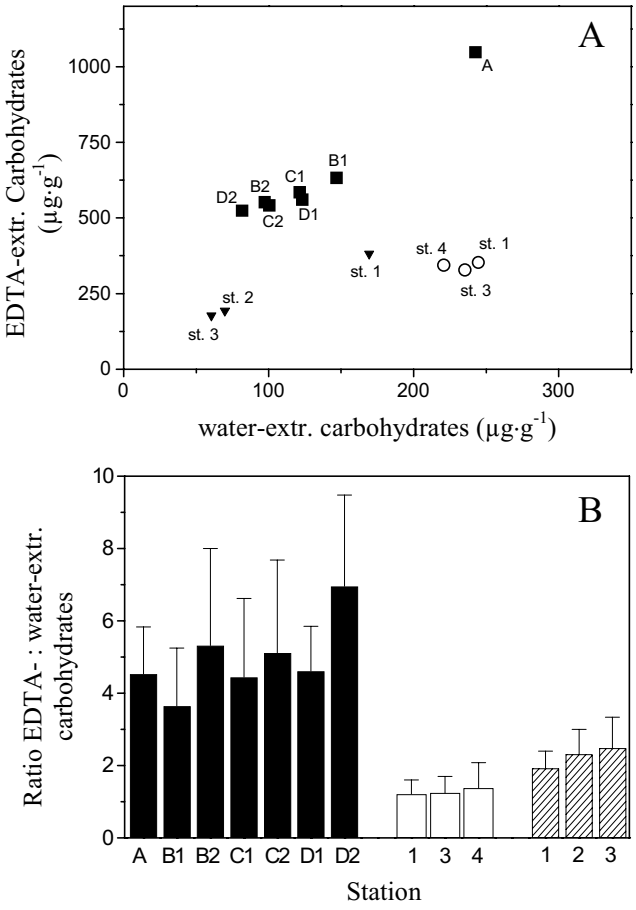

Figure 8.2. Depth averaged carbohydrate content (A), and ratio of water-extractable to EDTA-extractable carbohydrates (mean ± 1 SD) (B) in the Humber mudflat (■, black bars), the Marennes mudflat (o, white bars) and the Dollard mudflat (▼, hatched bars).

where ridge-runnel structures were the dominant morphological feature. The differences observed at station 1 in the Dollard could be explained in terms of grain size. Station 1 consisted of muddy sediment with a median grain size of 26 µm whereas station 2 and 3 were more sandy with a median grain size of 59-91 µm. In the Humber and the Dollard mudflats contents of both carbohydrate fractions decreased in a seaward direction. This was not the case in the Marennes mudflat. The ratios of EDTA- to water-extractable carbohydrate differed significantly between the different mudflats ($F_{(2, 40)}$= 239.7; $p<0.001$). Ratios were close to 1 in the Marennes mudflat whereas it varied between 3.7 and 7 in the Humber mudflat, reflecting a large contribution of EDTA-extractable carbohydrates (Fig. 8.2B). Ratios for the Dollard mudflat were in between those reported for the Marennes and the Humber mudflat and varied between 1.9 and 2.3. In the Humber mudflat, carbohydrate contents for both fractions were lower in the runnels compared to the ridges (Fig. 8.2A, compare stations denoted 1 and 2). Ratios were typically higher in the runnels ($F_{(3, 24)}$=4.0; $p<0.05$, Fig. 8.2B) indicating that in these features water-extractable carbohydrates were depleted compared to EDTA-extractable carbohydrates.

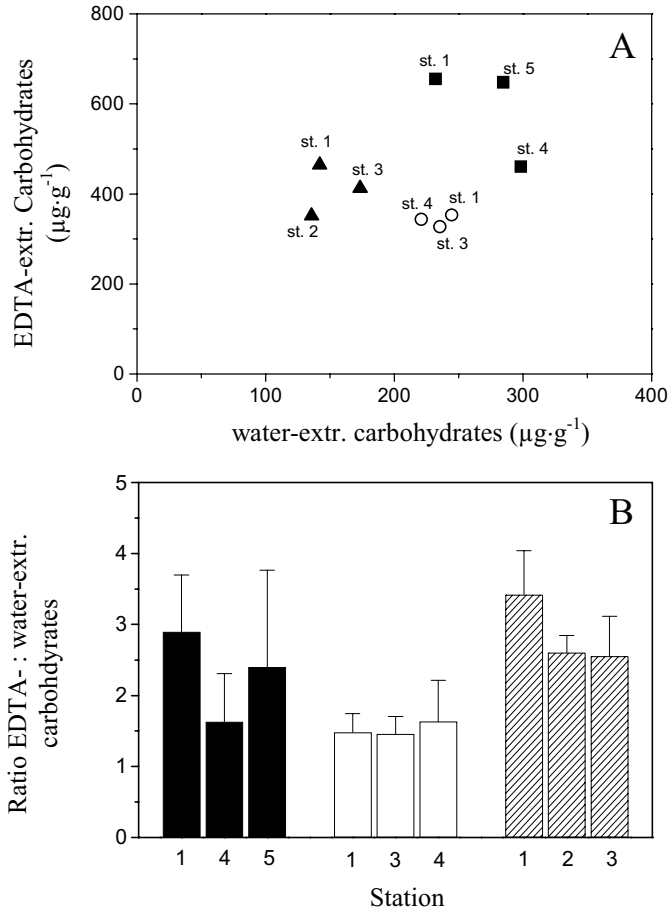

Figure 8.3. Depth averaged carbohydrate content (A) and ratio of water-extractable to EDTA-extractable carbohydrate (mean ± 1 SD) (B) in the Marennes mudflat, November 1996 (■, black bars), April 1997 (o, white bars) and November 1997 (▲, hatched bars).

The spatial patterns in extracellular carbohydrate contents that were observed between various mudflats were also observed when the Marennes mudflat was sampled on a temporal basis (Fig. 8.3). The stations sampled within a sampling campaign clustered together (Fig. 8.3A). The ratios between the carbohydrate fractions differed between stations ($F_{(6, 18)}$=16.4; p<0.001) reflecting the variation along the transect. Ratios also differed between the sampling campaigns ($F_{(2, 18)}$=8,2; p<0.05) showing the variation in carbohydrate content at a temporal scale.

Monosaccharide distribution

Monosaccharide distributions showed little dynamics with depth and therefore the results for the different depth intervals were averaged (Table 8.2, 8.3, 8.4). Monosaccharide distributions were roughly constant among stations as well as between the different mudflats. The monosaccharide distribution of the water- and EDTA-extractable carbohydrate fractions was somewhat different. In both carbohydrate fractions galactose was present in high relative amounts. However, on average the contribution of this monosaccharide was higher in the EDTA-extractable carbohydrate fraction and differed about 5 % between the two fractions (20.1± 4.8 % and 25.1± 2.7 %, for the water- and EDTA-extractable fraction, respectively). In contrast, the average relative contribution of rhamnose decreased from 18.1±4.7 % in the water extractable carbohydrate fraction to 13.1±2.6 % in the EDTA-extractable fraction.

Table 8.2. Depth averaged monosaccharide distribution of water- and EDTA-extractable carbohydrates in the Humber mudflat. Values in parenthesis indicate standard deviations (n=6).

Station	fucose	rhamnose	arabinose	galactose	glucose	xylose/ mannose	galacturonic acid	gluceronic acid
				Weight %				
water-extractable carbohydrates								
A	9.7 (1.8)	24.0 (3.5)	6.5 (0.4)	18.4 (2.0)	12.1 (0.5)	21.3 (1.0)	3.5 (3.2)	4.4 (4.1)
B_1	8.4 (1.3)	23.6 (3.3)	6.4 (3.7)	19.5 (1.6)	11.0 (6.4)	19.1 (8.5)	5.9 (4.4)	6.0 (2.2)
B_2	9.8 (1.5)	19.6 (0.9)	7.3 (0.8)	17.4 (1.5)	16.3 (5.5)	21.9 (1.8)	3.3 (0.6)	4.4 (0.5)
C_1	9.7 (0.9)	21.3 (1.5)	7.6 (0.7)	17.9 (0.8)	12.7 (1.3)	22.4 (0.7)	3.6 (0.2)	4.8 (0.5)
C_2	6.1 (0.5)	22.6 (2.7)	5.7 (0.7)	19.2 (1.2)	10.6 (1.6)	23.6 (1.4)	4.9 (0.9)	7.3 (0.7)
D_1	9.1 (1.6)	19.5 (0.9)	9.8 (1.0)	18.9 (1.5)	12.3 (0.5)	23.6 (1.8)	3.0 (1.7)	3.8 (2.2)
D_2	4.7 (3.0)	20.7 (1.0)	5.6 (2.0)	20.5 (2.5)	12.4 (2.3)	23.5 (1.6)	4.3 (2.2)	8.3 (3.2)
EDTA-extractable carbohydrates								
A	2.7 (1.3)	17.8 (2.6)	5.4 (0.6)	22.1 (2.4)	14.1 (2.3)	23.3 (2.7)	6.5 (1.6)	8.1 (4.0)
B_1	9.3 (1.4)	13.7 (0.7)	8.7 (0.7)	24.1 (1.6)	14.2 (2.9)	23.8 (0.7)	2.5 (0.8)	3.7 (1.7)
B_2	13.0 (1.9)	12.3 (0.9)	6.5 (1.5)	24.3 (1.7)	14.2 (3.5)	22.6 (1.0)	3.6 (1.4)	3.4 (0.6)
C_1	12.6 (1.4)	12.8 (1.3)	7.1 (1.1)	25.1 (1.3)	12.1 (1.4)	23.4 (0.6)	3.7 (0.9)	3.1 (0.6)
C_2	12.9 (1.5)	14.2 (3.6)	7.7 (0.9)	27.5 (2.5)	12.9 (1.1)	15.3 (4.0)	5.1 (0.8)	4.1 (0.4)
D_1	8.1 (1.6)	11.0 (1.2)	7.9 (2.3)	24.6 (1.2)	11.4 (0.5)	20.1 (0.9)	9.5 (0.4)	7.4 (2.7)
D_2	9.3 (1.1)	12.3 (0.6)	6.9 (1.3)	25.7 (2.3)	13.7 (1.1)	22.8 (1.2)	4.4 (1.8)	5.0 (0.8)

Table 8.3. Depth averaged monosaccharide distribution of water- and EDTA-extractable carbohydrates in the Marennes mudflat (April 1997). Values in parenthesis indicate standard deviations (n=6)

Station	fucose	rhamnose	arabinose	galactose	glucose	xylose/ mannose	galacturonic acid	gluceronic acid
				Weight %				
water-extractable carbohydrates								
st 1	14.0 (0.5)	16.6 (1.1)	5.9 (0.3)	20.5 (0.7)	6.8 (0.3)	21.1 (0.8)	7.5 (0.6)	7.7 (0.8)
st 3	11.7 (0.8)	14.1 (0.9)	6.2 (0.3)	21.4 (1.3)	12.6 (0.7)	21.7 (0.3)	5.2 (0.5)	7.0 (0.1)
st 4	11.3 (1.2)	13.6 (1.1)	6.2 (0.3)	20.1 (1.0)	12.3 (0.6)	22.6 (2.8)	6.0 (0.2)	7.9 (0.8)
EDTA-extractable carbohydrates								
st1	15.4 (1.7)	12.4 (1.1)	5.9 (0.7)	30.1 (3.1)	7.7 (1.4)	21.6 (1.9)	3.8 (4.6)	3.2 (4.4)
st3	11.0 (0.9)	10.1 (0.6)	5.8 (0.3)	24.9 (1.5)	12.8 (0.5)	20.7 (0.5)	6.9 (1.8)	7.7 (1.3)
st4	11.8 (0.9)	10.2 (0.4)	6.1 (0.3)	24.2 (0.9)	12.6 (0.5)	20.8 (0.4)	7.3 (1.5)	6.9 (0.4)

Partially different trends were observed in the Dollard mudflat, where the contribution of fucose and arabinose decreased with increasing median grain size. This coincided with an increasing contribution of uronic acids (Table 8.4). Xylose/mannose was present in high relative amounts and accounted on average for 20.1±4.8 %. Arabinose was found in minor amounts in all samples (6.5±2.1 %), while the contribution of fucose and and glucose was on average 9.4±3.7 % and 12.2±3.4 %, respectively. Uronic acids accounted for 11.4±5.5 % of the total.

Table 8.4. Depth averaged monosaccharide distribution of water- and EDTA-extractable carbohydrates in the Dollard mudflat. Values in parenthesis indicate standard deviations (n=6)

station	fucose	rhamnose	arabinose	galactose	glucose	xylose/ mannose	galacturonic acid	gluceronic acid
				Weight %				
water-extractable carbohydrates								
st 1	11.9 (1.8)	18.5 (1.2)	6.1 (3.0)	22.9 (1.4)	13.4 (0.8)	17.3 (3.4)	5.2 (0.8)	4.8 (1.0)
st 2	7.5 (3.4)	19.4 (0.9)	5.9 (1.5)	26.8 (1.0)	14.8 (1.3)	12.0 (1.5)	6.1 (0.8)	7.6 (1.5)
st 3	3.6 (3.6)	16.6 (1.2)	3.0 (2.3)	25.3 (2.1)	7.5 (2.3)	21.7 (1.0)	9.6 (0.8)	12.8 (3.4)
EDTA-extractable carbohydrates								
st 1	12.2 (1.1)	16.5 (1.7)	9.0 (0.6)	25.8 (2.0)	14.4 (1.0)	12.1 (4.3)	6.3 (3.5)	3.6 (1.4)
st 2	9.4 (1.3)	15.8 (1.4)	8.0 (0.7)	26.0 (2.0)	13.4 (0.5)	12.2 (1.5)	9.2 (2.9)	6.0 (1.1)
st 3	6.5 (2.7)	11.7 (1.2)	6.1 (0.7)	21.1 (1.3)	13.0 (3.8)	20.2 (1.2)	12.5 (1.2)	8.9 (1.6)

Pigments

The pigment composition of the extracts from all sampled stations revealed a low contribution of chlorophyll *b* and zeaxanthine indicating that both cyanobacteria and green algae were of minor importance (data not shown). The high contribution of fucoxanthine indicated that the major part of the phototrophic community (measured as chlorophyll *a*) was comprised of diatoms. Vertical chlorophyll *a* profiles showed decreasing contents with depth (see Fig. 8.1). Chlorophyll *a* contents at the surface 0.5 cm varied between 1.0-10.3 $\mu g \cdot g^{-1}$, and diatom patches were visible when contents exceeded 7 $\mu g \cdot g^{-1}$ (Table 8.1).

Grain size analysis

The distribution in grain size along transects differed between the three mudflats (Table 8.1). In the Humber mudflat median grain size steadily increased from the shore

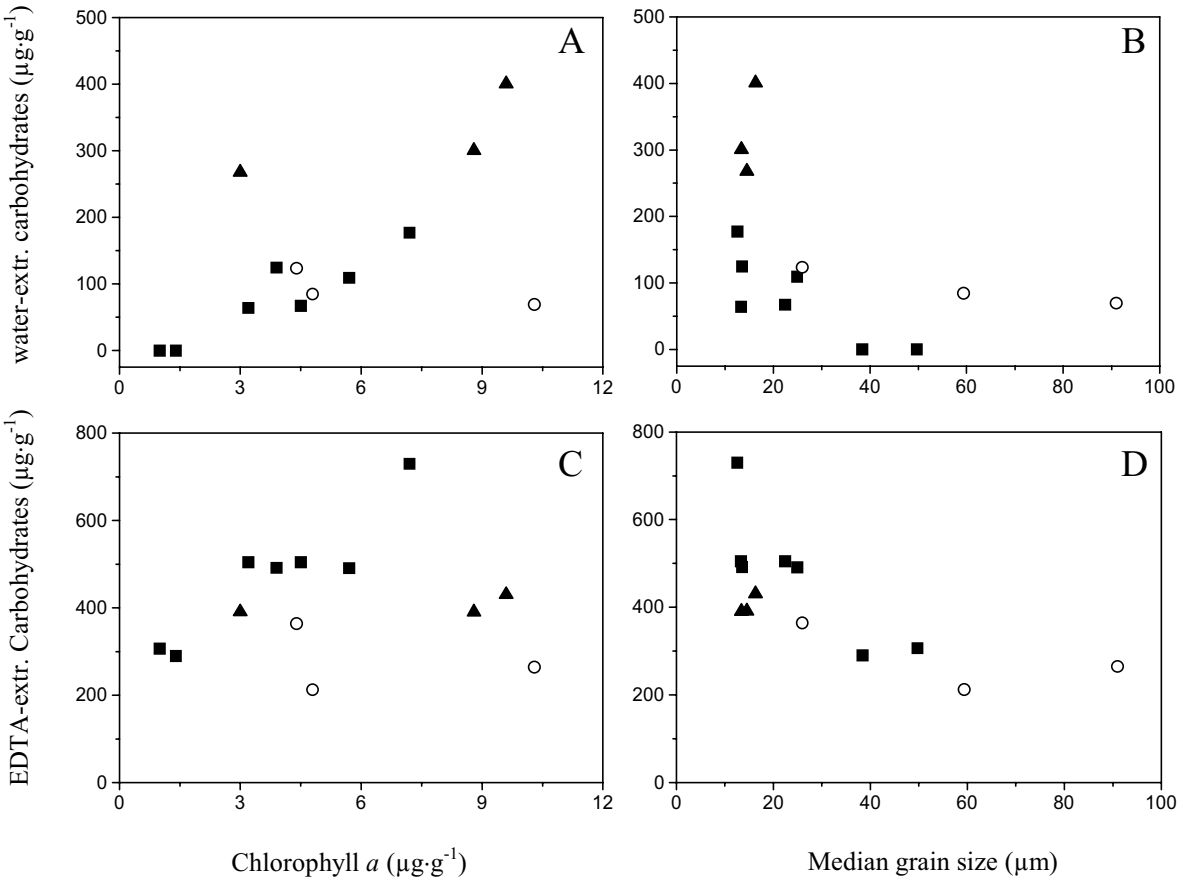

Figure 8.4. Relationships between water-extractable carbohydrate and chlorophyll *a* (A), water-extractable carbohydrate and median grain size (B), EDTA-extractable carbohydrate and chlorophyll *a*

(C) and EDTA-extractable carbohydrate and median grain size (D). Results of the top 0.5 cm were used. Data were from the Humber mudflat (■), the Marennes mudflat (▲) and the Dollard mudflat (o)

towards the low water line. In the Dollard mudflat a similar pattern was observed although on average the sediment was coarser. In contrast, the grain size distribution in the Marennes mudflat was constant between the stations that were sampled. The sediment was typically muddy with a median grain size of about 15 µm.

Relation between chlorophyll a, median grain size and carbohydrates

The majority of the benthic diatoms were confined to the surface sediment layer. Therefore, data from the top 0.5 cm of the sediment were taken to investigate the relations of the two carbohydrate fractions with chlorophyll *a* and the median grain size (Fig. 8.4). The water-extractable carbohydrate fraction showed a positive correlation with chlorophyll *a*, with station 3 in the Dollard mudflat and station 4 in the Marennes mudflat deviating from this trend. EDTA-extractable carbohydrates were not related to chlorophyll *a* contents. Both colloidal- and EDTA-extractable carbohydrate contents decreased with increasing median grain size.

Incubation experiment

The results of the incubation experiment are shown in Fig. 8.5. Changes in carbohydrate contents were observed in the top 0.5 cm sediment layer only. In the cores incubated in the light under exposed conditions water-extractable carbohydrate contents increased threefold (compared to carbohydrate contents in the field (Fig. 8.1A)), while EDTA-extractable carbohydrate content increased twofold. In the immersed, light incubated cores, production of carbohydrate was evident in both fractions although to a lesser extend than in the exposed cores. Water-extractable carbohydrates increased with 25 % in the dark exposed cores while EDTA-extractable carbohydrate was constant. In contrast, EDTA-extractable carbohydrate increased in the dark immersed cores. This was however balanced by the decrease in water-extractable carbohydrates.

Chlorophyll *a* contents in the top 0.5 cm sediment layer were highest in the exposed cores with contents of 10.6 and 12.9 $\mu g \cdot g^{-1}$. In the deeper sediment layers contents were roughly constant (Fig 8.5A, B). In contrast, chlorophyll *a* contents in the immersed cores were lower in the top 0.5 cm, and chlorophyll *a* contents gradually decreased with depth (Fig. 8.5C, D). The depth integrated chlorophyll *a* content was highest in the light incubated, exposed sediment with a content of 3.4 $\mu g \cdot g^{-1}$. In the dark incubated cores this was 2.9 and 2.7 $\mu g \cdot g^{-1}$

for the exposed and the immersed cores, respectively. The lowest value was found in the light incubated immersed cores (2.3 µg·g^{-1}).

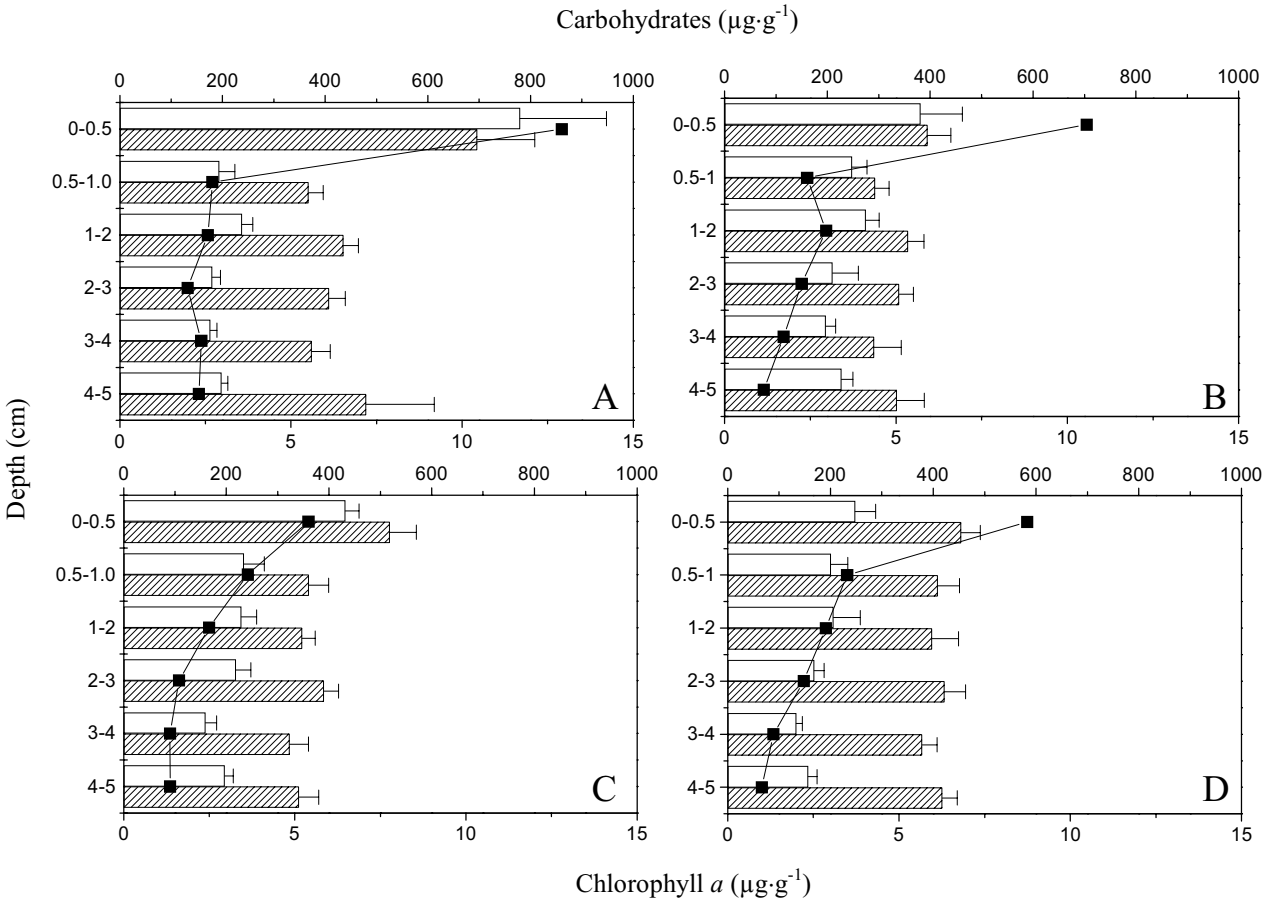

Figure 8.5. Vertical profiles of water-extractable carbohydrate (white bars, mean ± 1 SD), EDTA-extractable carbohydrate (hatched bars, mean ± 1 SD) and chlorophyll *a* profiles (■) after 48 h incubations of sediment cores (Marennes mudflat, station 4) in the light, emersed (A), in the dark, emersed (B), in the light immersed (C), in the dark immersed (D).

Discussion

The results presented in this study indicate that dynamics in the contents of extracellular carbohydrates may take place over larger areas, typically over the scale of a mudflat. This was clear from the datasets of the spatial as well as the temporal distribution of carbohydrates in the different intertidal mudflats that were investigated. This indicates that for different mudflats that were sampled the processes that determined the contents of carbohydrates in intertidal sediments were acting on similar scales. Benthic diatoms are

considered to represent an important source of carbohydrates as 40-70 % of fixed carbon may be excreted into the environment as extracellular sugars (Goto et al. 1999; Middelburg et al. 2000; Smith & Underwood 2000; chapter 5). The incubation experiment conducted in this study showed that production of extracellular carbohydrates was primarily related to photosynthesis with the highest production measured in the water-extractable carbohydrate fraction. Assuming that in the dark under exposed conditions biomass was constant over the incubation period, an increase of 17 % in chlorophyll *a* (over the 5 cm depth layer) was observed when sediments were incubated in the light under emersed conditions. This shows that under these conditions photosynthetically fixed carbon was partitioned between both balanced and unbalanced growth. When cores were incubated in the dark under immersed conditions a decrease of 7 % in chlorophyll *a* was observed. A higher amount of 22 % of the chlorophyll *a* was removed from the sediment when cores were incubated in the light and under immersed conditions. This probably reflects active migration of the microphytobenthos towards the light, after which part of the diatoms is transferred into the overlying water. It is known that benthic diatoms exhibit migratory rhythms in response to diel and tidal cycles (Serôdio et al. 1997). Our results however suggest that the migratory behavior of diatoms under prolonged periods of light is not related to these migratory rhythms. Production of extracellular carbohydrate under light, immersed conditions was substantially lower compared to the light, exposed situation. This was probably a combined effect of i) lower light levels reaching the sediment surface due to the overlying water, ii) decreases in diatom biomass during the incubation, iii) dissolution of carbohydrate into the overlying water. In comparison to the light related carbohydrate production, contents of extracellular carbohydrates increased only marginally during the dark incubations. Possibly this production was related to diatom motility (Edgar & Pickett-Heaps 1984; Smith & Underwood 1998) which involves the excretion of extracellular polysaccharides.

Underwood & Smith (1998b) presented a simple relationship between chlorophyll *a* and water-extractable carbohydrate contents in intertidal sediments that was valid for intertidal mudflats where epipelic diatoms constituted >50% of the microphytobenthos assemblage. A comparable relationship was found in this study (Fig. 8.4A), which explained 35 % of the variability in the surface 0.5 cm of the sediment. In chapter 7,the relation between chlorophyll *a* and water-extractable carbohydrates in the Humber mudflat was explored and it was concluded that the strength of this relationship was dependent on various other parameters including sediment characteristics and the geomorphological structures within the mudflat.

Measurements of the median grain size of the sediments were related to the carbohydrate content for both water- and EDTA-extractable carbohydrates (Fig 8.4B, D). This is consistent with other studies (Paterson et al., 2000; chapter 6) that reported significant relations between extracellular carbohydrates and grain size using nonparametric tests. Also Bergamaschi et al. (1997) found that sugar contents were related to sediment grain size by

separating bulk sediment of the Peru continental margin into different size classes. These results indicate that apart from the production of carbohydrate by microphytobenthos, grain size distribution plays a role as a determinant of the carbohydrate content in intertidal sediments. Hydrodynamic processes such as tidal currents and wave action govern sediment transport and affect the grain size distribution of the sediment), thereby changing the characteristics of the sediment surface (Christie et al. 1999; chapter 6). During storm events sediment erosion may occur while during calm weather conditions sediment is deposited upon the mudflat (Christie et al. 1999). These hydrodynamic processes influence relatively large areas such as whole mudflats and may result in resuspension of microphytobenthos and sediment transport (de Jonge & van Beusekom 1995; Christie et al. 1999). Because both chlorophyll *a* and sediment grain size are related to the carbohydrate content (Fig 8.4) it seems likely that spatial patterns in the extracellular carbohydrate distribution as were found in this study are at least to some extent determined by the hydrodynamic regime acting upon the intertidal areas. This is in agreement with observations of Dyer et al. (2000) who found that wave exposure is one of the primary external variables driving the morphodynamics of intertidal mudflats.

Monosaccharide distributions of the carbohydrate fractions were constant to a depth of 5 cm. Cowie & Hedges (1984) observed that the carbohydrate composition in a sediment core taken from a coastal marine environment was constant to a depth of 50 cm. This was taken to be an example of an advanced stage of carbohydrate decomposition. The carbohydrate distributions that were analyzed by us closely resembled the composition of the carbohydrate component of marine DOM found in surface seawaters (McCarthy et al. 1996; Aluwihare et al. 1997; Aluwihare & Repeta 1999) and alkali extractable polysaccharides in marine sediments (Miyajima et al. 2001). For dissolved organic matter the uniformity in the composition of polysaccharides was a result of rapid removal of a bioreactive part of the carbohydrates by bacteria, while the refractory carbohydrate pool that remained showed little variation over large spatial scales (Aluwihare et al. 1997; Amon et al. 2001). Similar mechanisms may govern the constancy of the carbohydrate composition that were found in this study (see also Miyajima et al. 2001). Middelburg et al. (2000) observed that transfer of photosynthetically fixed carbon from algae to bacteria was rapid and probably a result of the bacterial utilization of exudates produced by the algae. In addition, it was observed that microphytobenthos excreted glucose-rich polysaccharides in the light when the sediment was exposed (chapter 5). This freshly produced carbohydrate was removed during the subsequent immersion period leaving behind a carbohydrate fraction that was more heterogeneous in monosaccharide distribution. This suggests that freshly produced carbohydrates in mudflats may be turned-over over a time-scale of hours. In marine coastal sediments it was calculated that, potential carbohydrate hydrolysis rates were in the same order of magnitude (Arnosti & Holmer 1999).

Although monosaccharide distribution appeared to be comparable over large spatial scales there was a consistent difference between the two carbohydrate fractions that were sampled (Table 8.2). This was mainly reflected in the contributions of rhamnose and galactose that differed substantially between the two carbohydrate fractions. Apart from the compositional dissimilarity between the carbohydrate fractions, adsorption characteristics under varying Ca^{2+}-concentrations were different (chapter 9). This indicates that the operational fractionation between water- and EDTA-extractable carbohydrates yields two distinctive groups of carbohydrates present in sediments rather than being subgroups of one sedimentary organic matter pool (Thimsen et al. 1998). This was also found in other studies where the composition of dissolved and adsorbed carbohydrates fractions were compared (Sigleo 1996; Taylor et al. 1999). The reasons for these differences (for example, whether they are originating from different carbohydrate sources or whether they represent carbohydrates in a different state of degradation) remain to be investigated.

The distribution of carbohydrate contents in intertidal sediments is the result of multiple interacting processes (Christie et al., 1999; chapter 6). It is therefore unlikely that the correlations that were found in this study are straightforward. For example, microphytobenthos may actively alter surface sediment properties by binding fine-grained sediments through the excretion of extracellular products (Frostick & McCave 1979; chapter 6). In turn, diatoms support higher growth rates in silty sediments. This provides a positive feedback between biology and sedimentology (Staats et al. 2001; van de Koppel et al. 2001). Hence, increases in carbohydrate contents in intertidal mudflats are due to both biological and sedimentological factors. In addition, results on the monosaccharide distribution analyzed in three intertidal mudflats indicate that this material consists of biorefractory material rather that carbohydrate that is freshly produced by benthic diatoms (see discussion above). Therefore, a major part of extracellular carbohydrate produced by microphytobenthos was removed or altered which may have been due to heterotrophic utilization (van Duyl et al. 1999, Goto et al. 2001), dissolution into the overlying water (Underwood & Smith 1998b, de Winder et al. 1999) or sorption to sediment particles (Hedges & Keil 1995). Also, other sources of carbohydrate may contribute to the sediment carbohydrate contents. For example, Galois et al. (2000) argued that part of the carbohydrates found in sediment of the Marennes mudflat originated from mucus-rich mollusc biodeposites. The importance of these processes is at the moment largely unknown and it remains to be investigated to what extend the observed correlation reflects a direct effect of diatoms on carbohydrate contents or whether it is a result of different processes acting in a similar fashion upon the two variables.

CHAPTER 9

SORPTION OF EPS TO SEDIMENT PARTICLES AND THE EFFECT ON THE RHEOLOGY OF SEDIMENT SLURRIES

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Abstract

Extracellular Polymeric Substances (EPS) are considered to play an important role in the stabilization of intertidal mudflats. In this study, the role of EPS as a binding agent in intertidal sediments was investigated. For this purpose two EPS fractions (termed coll-SF and EDTA-SF) were isolated from intertidal sediment and characterized in terms of monosaccharide- and size distribution. In slurry addition experiments the sorption characteristics of these EPS-fractions as well as their effect on sediment properties were examined under varying Ca^{2+}-concentrations. Results showed more EDTA-SF adsorbed to the sediment compared to coll-SF. For both fractions more EPS adsorbed to the sediment when Ca^{2+}-concentration increased. This effect was stronger for EDTA-SF. The differences in sorption between the two fractions could not be explained in terms of monosaccharide- and size distribution, which were largely similar. The addition of EPS in the presence or absence of Ca^{2+} did not alter the rheology of the sediment slurries indicating that there was no effect of EPS on the sediment properties. This contradicts results of experiments with bacterial EPS as well as field observations in which the presence of EPS/biofilms leads to an increase in the erosion resistance of the sediment. Possible causes for this discrepancy in results are discussed.

Introduction

Intertidal mudflats are highly dynamic areas and changes in the morphology are governed by complex interactions between biological and physical processes (Amos et al. 1998; Paterson & Black 1999; chapter 6). The organisms that make up the biological component in these environments are often able to modify surface sediment properties, thereby changing the sediment stability. This biological mediation may result in destabilization of the sediment, for example due to bioturbation by macrofauna (Paterson & Black 1999). On the other hand, many organisms including macrofauna (Meadows et al. 1990; Mouritsen et al. 1998), bacteria (Dade et al. 1990) and benthic diatoms (Underwood & Paterson 1993a; Sutherland et al. 1998) are able to stabilize the sediment. In general, stabilization of intertidal sediments has been related to the excretion of extracellular polymeric substances (EPS) by benthic organisms. The EPS may exert its stabilizing effect via grain to grain adhesion (Yallop et al. 1994; Paterson 1997), drag reduction (Paterson & Black 1999; de Deckere et al. 2001) or the formation of networks of EPS reinforced tubes (Meadows et al. 1990). Dade et al. (1990) directly showed the effect of bacterial EPS as a binding agent by adding polymers isolated from the bacterium *Alteromonas atlantica*. In cohesive sediments, information on the role of EPS in sediment stabilization comes solely from correlative studies in which benthic diatoms are considered to be the most important EPS producers (Kornman & de Deckere 1998; Tolhurst et al. 1999; Paterson et al. 2000). A predictive relationship was formulated by Amos et al. (1998) calculating the critical erosion threshold from bulk density and water-extractable carbohydrates. Yallop et al. (2000) also proposed a predictive relation that explained sediment stability in terms of water-extractable carbohydrate, chlorophyll *a* and water content. Although in these correlative studies EPS was considered to be important as a stabilizing agent, the effect of the EPS as such could not be established. Rather, the effect of the diatom biofilm as a whole is determined using these methods.

In this study the role of EPS as a binding agent in intertidal mudflats was addressed. For this purpose, bulk quantities of two commonly extracted EPS-fractions (Underwood et al. 1995; de Winder et al. 1999; chapter 6) were isolated from an intertidal mudflat and subsequently used in slurry addition experiments. These EPS fractions were used to study the sorption of EPS to sediments under varying Ca^{2+}-concentrations. Divalent cations like Ca^{2+} and Mg^{2+} play a role in the interactions between exopolymers and mineral surfaces and between the exopolymers themselves (Tipping 1981; Decho 1994). In colonies of *Phaeocystis* it was observed that these cations were essential for the gelling of the colony mucus while potassium showed no effect (van Boekel 1992). The effect of EPS as an adhesive in intertidal sediments was examined using rheological methods. James et al. (1988) stated that knowledge of the rheological properties of cohesive sediments is important in relation to sediment

transport processes. In addition, Ruddy et al. (1998a) showed that changes in sediment structure (e.g. erosional events) at the sediment-water interface could accurately be followed using rheological methods. The dynamic viscosity, fluidity and rigidity of the sediment slurries were measured, providing data on the visco-elastic properties of the sediment slurries.

Material and methods

Isolation of EPS fractions

Carbohydrate material for the addition experiments was isolated from sediment collected from Banc de St-Vivien, an intertidal mudflat situated in the Gironde estuary, France. The surface layer of the sediment including to a depth of approximately 2 mm was sampled and lyophilized prior to the extraction procedures. At the time of sampling extensive diatom biofilms were not visible and chlorophyll *a* concentrations varied between 10.8 and 39.0 $mg \cdot m^{-2}$. Production of extracellular carbohydrate occurred during emersion and contents increased on average with 27% during exposure of the mudflat. Although extracellular carbohydrates may originate from different sources (including diatoms, bacteria, meio- and macrofauna) it was assumed that the extracellular carbohydrates that were isolated mainly originated from benthic diatoms (see also Underwood & Smith 1998b; chapter 7). It should however be noted that the fractions that were sampled may to some extend originate from other sources than benthic diatoms (see Yallop et al. 2000). Two operationally defined carbohydrate fractions were isolated according to procedures described in chapter 6. In short, the water-extractable fraction was obtained by extracting the lyophilized sediment with distilled water (1 h, 30°C). Subsequently, the sediment was extracted with 0.1 M Na_2-EDTA (16 h, 20°C) yielding the EDTA-extractable fraction. Both fractions were filtered through a 0.2 µm filter to remove the remaining particulate matter. Subsequently, the fractions were filtered through a 1000 dalton filter (Amicon) using a tangential-flow filtration set-up (Amicon). During this procedure the sample volumes were reduced to 100 ml after which the samples were diafiltered with 1 l milli-Q water to remove inorganic salts and low molecular weight carbohydrates. Finally, the sample volumes were reduced to 50 ml and the concentrates lyophilized. The carbohydrates that were isolated using this procedure had a molecular size > 1000 dalton, which means that the smallest sugars consisted of 5.6 sugar monomers (glucose equivalents). Because these fractions were devoid of monosaccharides and small oligosaccharides that may pass cell membranes (<600 dalton, Weiss et al. (1991)) they were designated EPS. The water-extractable EPS (which in literature is often referred to as colloidal EPS) that was isolated and purified in this way was termed coll-SF (colloidal

Sugar Fraction), while EDTA-extractable EPS was termed EDTA-SF (EDTA-extractable Sugar Fraction). For both fractions the lyophilized isolates were pooled and dissolved in 500 ml milli-Q water.

Characterization of the EPS fractions

The EPS fractions were characterized for their size distributions by means of ultrafiltration using Centricon centrifuge tubes (Millipore) with molecular weight cut-off filters of 100, 50 and 10 kdalton (chapter 5). In all size fractions the monosaccharide distribution was analyzed by HPLC-PAD. Prior to analysis the polymers were hydrolyzed and converted to their monosaccharides by means of acid methanolization followed by trifluoroacetic acid-hydrolysis (chapter 5).

Sorption experiments

Sediment collected from Banc de St-Vivien in the Gironde estuary, France was lyophilized, sieved over a 280 µm mesh sieve and the sediment fraction < 280 µm was used as the solid phase in the sorption experiments. No further manipulations were carried out in order not to disturb the sediment. For the sorption experiments slurries were prepared with a solid to solution ratio of 333 $g \cdot l^{-1}$. This is equivalent to a water content of 75 % which is within the range of what is commonly found in surface sediments in the presence of diatom biofilms (chapter 6). The slurries were prepared using 4 different EPS contents (1313.6 and 2627.1 $\mu g \cdot g^{-1}$ coll-SF, 821.3 and 1642.5 $\mu g \cdot g^{-1}$ EDTA-SF) and a control without EPS added. For each EPS addition the Ca^{2+}-contents were varied between 0-300 $\mu mol \cdot g^{-1}$ by adding $CaCl_2$. The ionic strength was kept constant at a value of 0.3 $mol \cdot kg^{-1}$ by the addition of NaCl. For each treatment five replicates were taken. The slurries were shaken for 12 h at 20°C and subsequently frozen and lyophilized. The partitioning of EPS was determined by extracting the sediment as described above. The EPS that was recovered in this way after the incubations are termed water- and EDTA-extractable EPS (note the difference in terminology with the EPS that was added at the start of the incubation experiment, which are called coll-SF and EDTA-SF). Carbohydrate contents in the water- and EDTA-extractable fractions were analyzed using the phenol-sulfuric acid method (Dubois et al. 1956). From these data the percentage of EPS in the colloidal, the EDTA-extractable fraction could be calculated. The difference between the amount of EPS (coll-SF or EDTA-SF) added at the start of the incubations and the amounts recovered in the water- and EDTA-extractable fractions after the incubations was operationally defined as the irreversibly adsorbed EPS fraction. The

adsorption data were fit with a Freundlich constant partitioning model:

$$C_s=K_dC_l \quad (1)$$

where C_s is the adsorbed carbohydrate content, C_l is the dissolved carbohydrate concentration and K_d is the partition coefficient for the EPS. Two partitioning coefficients were calculated. One for the partitioning of EPS between the dissolved and the adsorbed phase ($K_{d\text{-}coll}$) in which the adsorbed phase is the sum of the EDTA-extractable and irreversibly bound EPS, and the other for the partitioning of adsorbed EPS between the EDTA-extractable and the irreversibly bound fractions ($K_{d\text{-}EDTA}$). K_d-values were determined using linear regression analysis.

Rheological experiments

The rheological properties of sediment slurries were measured using a Carrimed controlled stress rheometer equipped with a concentric cylinder geometry with a gap width of 2 mm. The properties of the sediment slurries were examined using two modes of operation. First, the apparent viscosity of the sediment slurries was measured as a function of decreasing shear strength. Secondly, the visco-elastic properties of the sediment slurries were measured using variable oscillation frequency with an amplitude of 4 milliradians. This mode of operation yields two independent rheological parameters: the storage modulus G' and the dynamic viscosity n'. G' characterizes the elastic while n' characterizes the viscous contribution to the visco-elastic properties of the sample. Tan Δ is an indicator of the structure of the material and is defined as:

$$\text{Tan } \Delta=n'\omega/G'$$

where ω is the frequency ($rad \cdot s^{-1}$). For a perfect elastic solid Tan Δ is 0 (Δ =0°) while for a Newtonian fluid tan Δ is infinite (Δ=90°).

The effect of EPS (coll-SF and EDTA-SF) on the structural characteristics of the sediment slurries was investigated using slurries with a sediment concentration of 700 $g \cdot l^{-1}$. The sediment was diluted with milli-Q water, a solution of EPS and a solution of EPS with 143 $\mu mol \cdot g^{-1}$ $CaCl_2$. The contents of coll-SF and EDTA-SF were 834 $\mu g \cdot g^{-1}$ and 522 $\mu g \cdot g^{-1}$, respectively. The slurries were incubated for 12 h. After measuring the rheological properties,

the slurries were lyophilized, extracted and subsequently analyzed for carbohydrates in the water- and the EDTA-extractable fraction (n=3).

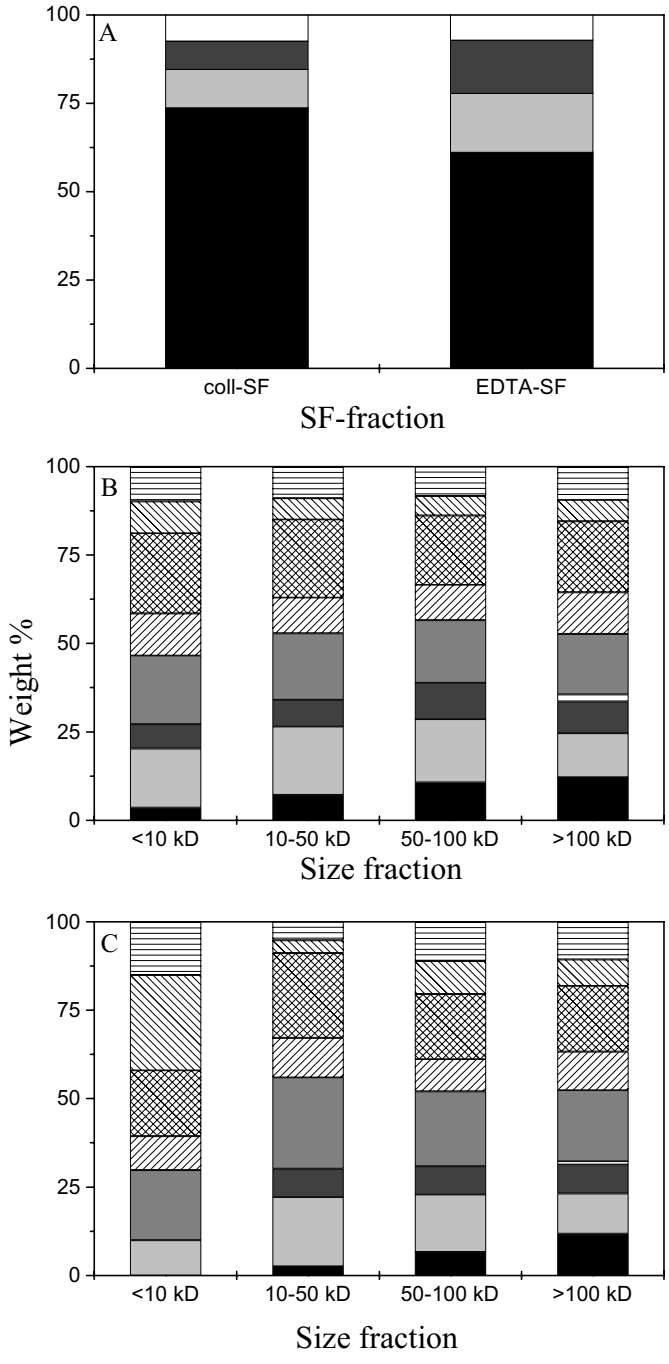

Figure 9.1. Size distribution of coll-SF and EDTA-SF (A), (: >100 kD, : 100-50 kD, : 50-10 kD, : 10-1 kD) and monosaccharide distribution of coll-SF (B) and EDTA-SF (C), (Fucose: , Rhamnose: , Arabinose: , Glucose-amine: , Galactose: , Glucose: , Xylose/Mannose: , Galacturonic acid: , Gluceronic acid: .

Results

Characterization of the EPS-fractions

The coll-SF and EDTA-SF were analyzed for their size and monosaccharide distribution (Fig. 9.1). Size fractionation experiments (Fig. 9.1A) showed that in both fractions polysaccharides in the size class >100 kD were most abundant and made up 73 and 62% of the total in the water- and EDTA-extractable fraction, respectively. In the size fractions of 1-10, 10-50 and 50-100 kD sugars were present in lower relative amounts and varied between 7 and 15%.

The monosaccharide distribution in the coll-SF was roughly constant when comparing the different size classes (Fig. 9.1B). EDTA-SF was more variable among the different size classes (Fig. 9.1C). In particular the 1-10 kD size class contained more uronic acids while arabinose was absent. The relative distribution of monosaccharides in the two EPS fractions was very similar. For the size classes >10 kD (93 % of the total EPS in both fractions) the difference in relative abundance of the various monosaccharides in the two EPS fractions were within 5% (except for galactose in the 10-50 kD size class, which differed 7%).

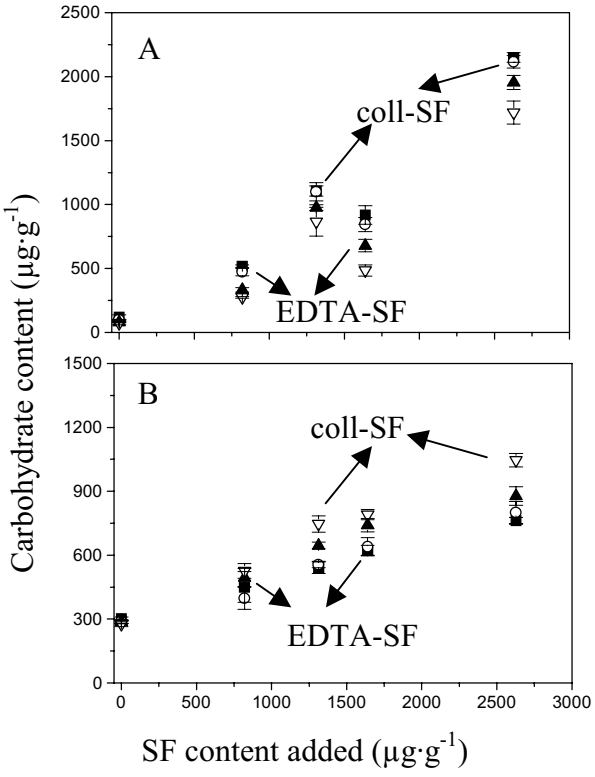

Figure 9.2. Partitioning of EPS (mean ± 1 SD) in the water-extractable carbohydrate fraction (A) and the EDTA-extractable carbohydrate fraction (B). Ca^{2+}-contents of 0 µmol·g^{-1} (■), 30 µmol·g^{-1} (o), 150 µmol·g^{-1} (▲), 300 µmol·g^{-1} (▽).

Table 9.1: Partitioning of coll-SF and EDTA-SF over the water-extractable, EDTA-extractable and irreversible fraction for the adsorption experiment. Values are given as the percentage of the total sugars added, corrected for blank adsorption. Values between brackets indicate standard deviations (n=5).

Ca^{2+} ($\mu mol \cdot g^{-1}$)	Coll-SF added 1313.6 $\mu g \cdot g^{-1}$	Coll-SF added 2627.1 $\mu g \cdot g^{-1}$	EDTA-SF added 821.3 $\mu g \cdot g^{-1}$	EDTA-SF added 1642.5 $\mu g \cdot g^{-1}$
Percentage of sugars in the water-extractable fraction				
0	84.5 (4.6)	77.3 (1.5)	48.3 (3.3)	48.6 (4.4)
30	85.7 (5.9)	76.8 (1.8)	45.6 (3.7)	45.4 (3.4)
150	76.7 (1.9)	71.4 (2.1)	30.8 (1.9)	36.5 (2.8)
300	68.1 (10.5)	62.7 (3.1)	24.7 (1.1)	25.1 (3.2)
Percentage of sugars in the EDTA-extractable fraction				
0	18.3 (1.6)	17.5 (0.8)	18.2 (9.1)	19.6 (1.5)
30	20.4 (1.3)	19.5 (1.8)	13.5 (7.1)	21.4 (2.3)
150	27.3 (0.7)	22.5 (1.7)	25.2 (5.0)	27.6 (2.1)
300	35.8 (2.6)	29.3 (1.5)	30.4 (5.0)	31.4 (1.7)
Percentage of sugars irreversibly adsorbed to the sediment				
0	-2.8 (3.7)	5.2 (1.5)	33.5 (10.7)	31.8 (3.9)
30	-6.2 (6.5)	3.7 (1.2)	40.9 (9.8)	33.2 (3.8)
150	-4.0 (1.9)	6.1 (2.5)	44.0 (5.3)	35.9 (1.4)
300	-3.9 (11.5)	8.0 (2.5)	45.0 (5.6)	49.7 (13.3)

Sorption experiment

The sorptive behaviour of the coll-SF and EDTA-SF fractions was investigated under varying Ca^{2+}-concentrations. The sorption was linear over the range of EPS-contents that were investigated (Fig. 9.2). Clear differences were observed between the sorption of coll-SF and EDTA-SF (Fig. 9.2, Table 9.1). For all Ca^{2+} treatments, the major part of the coll-SF was recovered in the water-extractable EPS-fraction (62.7-85.7 %), while this was 24.7-48.6 % for the EDTA-SF. In the EDTA-extractable fraction contents of coll-SF and EDTA-SF were comparable and varied between 13.5-35.8 %. Little of the coll-SF material was irreversibly adsorbed onto the sediment (-6.2-8.0 %). In contrast, the relative amount of irreversibly adsorbed EDTA-SF was substantial and varied between 31.8-49.7 %. The addition of Ca^{2+} clearly affected the sorption of the EPS fractions. The addition of 300 $\mu mol \cdot g^{-1}$ Ca^{2+} resulted

Table 9.2. Partitioning coefficients for coll-SF and EDTA-SF in the water-extractable and EDTA-extractable carbohydrate fractions under different Ca^{2+}-concentrations.

Ca^{2+} (µmol·g^{-1})	$K_{d\text{-coll}}$ (l·kg^{-1})		$K_{d\text{-EDTA}}$ (l·kg^{-1})	
	coll-SF	EDTA-SF	coll-SF	EDTA-SF
0	5.8 (0.2)	11.9 (0.6)	54.3 (0.9)	59.7 (5.5)
30	5.9 (0.2)	13.2 (0.6)	48.1 (1.9)	55.2 (5.5)
150	6.6 (0.2)	17.5 (0.9)	42.7 (1.9)	48.5 (2.4)
300	8.3 (0.4)	27.6 (1.3)	35.2 (1.9)	47.3 (1.8)

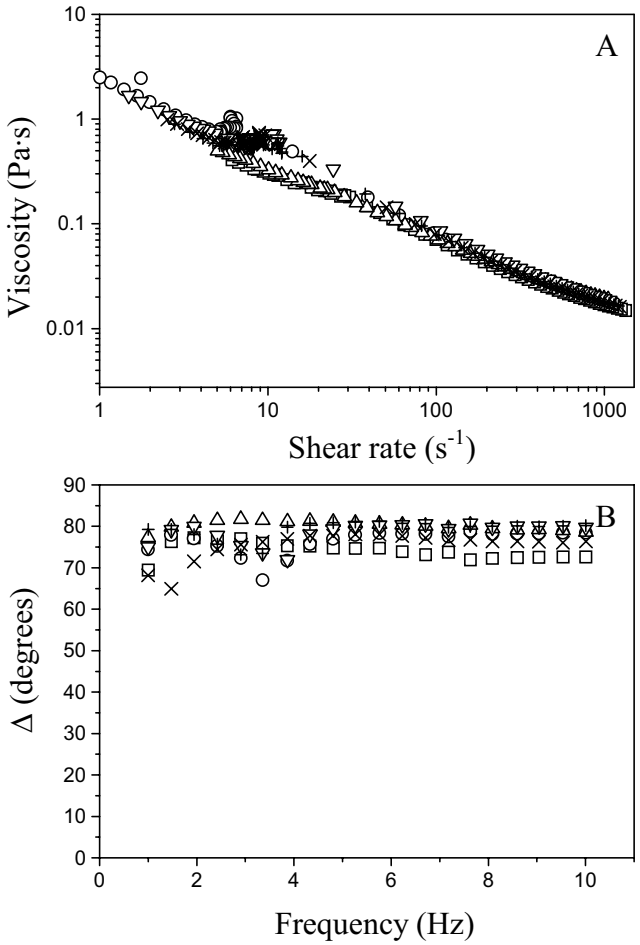

Figure 9.3. Flow curves (A) and variable oscillation frequency curves (B) for slurries prepared with mQ (□, ∇), coll-SF (o), coll-SF + Ca^{2+} (Δ), EDTA-SF (×), EDTA-SF + Ca^{2+} (+).

Rheological experiments

in increased sorption of the coll-SF and EDTA-SF with 15 and 24%, respectively (which was recovered either in the EDTA-extractable or irreversibly sorbed EPS fraction). The $K_{d\text{-}coll}$-values varied between 5.8-8.3 $l \cdot kg^{-1}$ and 11.9-27,6 $l \cdot kg^{-1}$ for coll-SF and EDTA-SF, respectively (Table 9.2). The addition of Ca^{2+} resulted in an increase in $K_{d\text{-}coll}$, which was higher for EDTA-SF (131 % increase at 300 $\mu mol \cdot g^{-1}$ Ca^{2+}) than for coll-SF (43 % increase at 300 $\mu mol \cdot g^{-1}$ Ca^{2+}). The $K_{d\text{-}EDTA}$-values give an indication of the partitioning of adsorbed matter between the EDTA-extractable and the irreversibly sorbed fraction. The addition of Ca^{2+} resulted in a decrease in $K_{d\text{-}EDTA}$, showing that the carbohydrate fraction that adsorbed under conditions of increasing Ca^{2+}-concentrations was preferentially bound in the EDTA-extractable fraction.

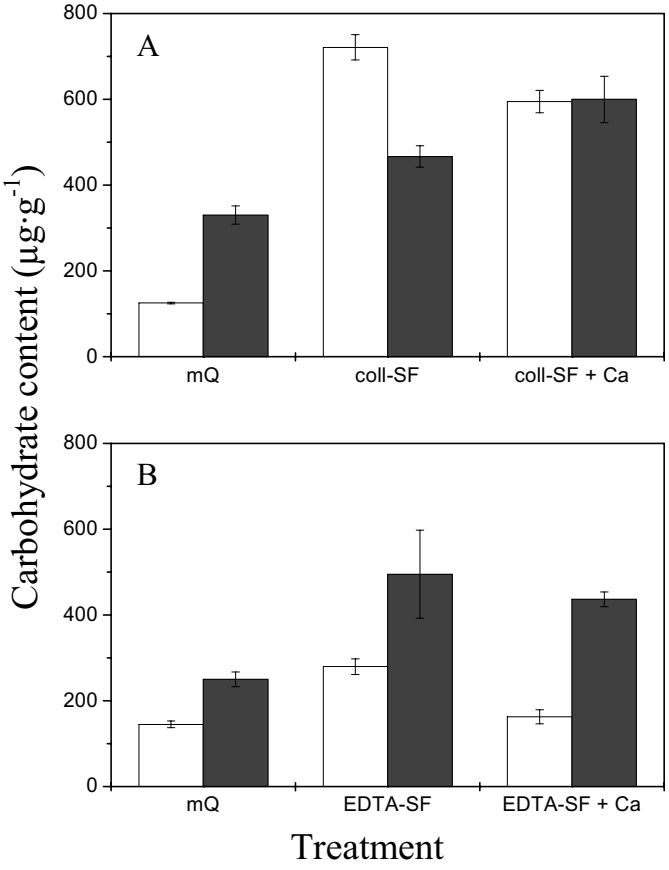

Figure. 9.4. Carbohydrate contents in slurries used for the rheology experiments with coll-SF (A) and EDTA-SF (B). White bars: water-extractable carbohydrates, grey bars: EDTA-extractable carbohydrates. Error bars indicate standard deviations (n=3).

Table 9.3: Partitioning of coll-SF and EDTA-SF over the water-extractable, EDTA-extractable and irreversibly adsorbed carbohydrate fractions for the rheological experiments. Values are given as the percentage of the total sugars added, corrected for blank adsorption. Values between brackets indicate standard deviations (n=3).

Treatment	Water-extractable fraction	EDTA-extractable fraction	Irreversibly adsorbed fraction
834 $\mu g \cdot g^{-1}$ coll-SF added			
Coll-SF	71.4 (3.5)	16.4 (3.0)	12.2 (4.6)
Coll-SF + Ca	56.3 (3.1)	32.3 (6.5)	11.4 (7.1)
521.9 $\mu g \cdot g^{-1}$ EDTA-SF added			
EDTA-SF	25.8 (3.5)	35.6 (1.3)	40.2 (8.6)
EDTA-SF + Ca	3.4 (3.1)	35.7 (3.3)	60.9 (2.2)

Results of the rheological experiments are shown in Fig. 9.3. Flow curves for the different slurries were identical and no effect of the addition of EPS (either coll-SF or EDTA-SF) in the presence or absence of Ca^{2+} was detected. Similar results were obtained for the oscillation experiments. Δ values for all treatments were close to 80 degrees. Therefore, the slurries behaved as viscous, inelastic fluids.

Carbohydrate contents for the additions of coll-SF and EDTA-SF are shown in Fig. 9.4. Clear differences in sorption characteristics were observed between the addition of coll-SF and EDTA-SF. The major part of the coll-SF that was added was recovered in the water-extractable fraction (71.4 %, Table 9.3). The addition of Ca^{2+} resulted in extra sorption of this material. Compared to coll-SF, a substantially lower part of the EDTA-SF was recovered in the water-extractable fraction (25.8 %, Table 9.3). This water-extractable material was bound to the sediment when Ca^{2+} was added (Table 9.3).

Discussion

In this study, distinct differences were observed in the sorption characteristics of two EPS-fractions that were isolated from an intertidal mudflat. The partition coefficients for water-extractable EPS ($K_{d\text{-}coll}$) varied between 5.8-8.3 $l \cdot kg^{-1}$ for the coll-SF, while EDTA-SF adsorbed more strongly to sediment particles with $K_{d\text{-}coll}$-values being 2-3 times higher (11.9-

27.6 $l \cdot kg^{-1}$). Arnarson & Keil (2000) studied the adsorption of easily extractable natural organic matter (NOM) to montmorrillonite. Using slurries with a comparable particle concentration (280 $g \cdot l^{-1}$), these workers showed that the NOM-fractions measured possessed K_d's that varied between 1.6-2.7 $l \cdot kg^{-1}$. In our study, the $K_{d\text{-}coll}$-values for coll-SF were somewhat higher which was possibly because the dissolved phase that determines the K_d' is defined differently between the two studies. $K_{d\text{-}coll}$-values for EDTA-SF were substantially higher compared to values found by Arnarson & Keil (2000) indicating a higher affinity of this organic matter fraction for sediment surfaces.

The difference in the sorptive behavior was also reflected in the response of the two EPS-fractions to varying Ca^{2+}-contents. A higher relative amount of the EDTA-SF adsorbed with increasing Ca^{2+}-content. Also, the adsorbed coll-SF was recovered mainly from the EDTA-extractable carbohydrate fraction while the major part of adsorbed EDTA-SF was irreversibly bound to the sediment. Henrichs (1995) stated that biologically produced polymers might bind to sediments via site specific adsorption. Irreversible adsorption then occurs when the polymer is attached to the sediment at many sites per molecule (Podoll et al. 1987). Our results indicate that Ca^{2+}-ions contributed to the sorption of EPS probably through the formation of cation bridges (Tipping 1981).

The clear differences in sorption between the coll-SF and the EDTA-SF suggest that these EPS-fractions are chemically different. Therefore, coll-SF and EDTA-SF were characterized in terms of monosaccharide composition and size distribution. As was shown in Fig. 9.1, both EPS-fractions were similar. Therefore, the difference in sorptive behavior between the two fractions could not be explained in terms of these compositional parameters. Structures of microbially excreted polysaccharides are generally complex and their physical behavior depends on various properties of the molecule. For example, the arrangement of monosaccharides within a molecule, linkage types and interactions with inorganic molecules have an effect on the properties of the EPS (Decho 1994) and could influence the sorption to sediment surfaces. Dade et al. (1990) reported that erosion resistance of sandy sediments increased with increasing uronic acid content of the polymers. In our study, a relation between EPS-sorption and the uronic acid content was not found but other negatively charged moieties like sulfated sugars and ketal-linked pyruvate groups could also be involved in the process of cation bridging (Sutherland 1990). These compounds were not measured in this study.

EPS excreted by benthic diatoms is often considered to act as an adhesive that binds sediment particles together in intertidal mudflats. In this way, sediment properties are modified resulting in an increased stability of the surface sediment layer. In our experiments, the addition of the EPS isolated from an intertidal mudflat did not alter the rheological properties of the sediment (Fig. 9.4). Flow curves for slurries incubated with EPS in the presence or absence of Ca^{2+} were similar to the blank slurries, while oscillation experiments showed that little structure was present. This contradicts results from Dade et al. (1990) and

Tolhurst et al. (personal communication) who found that the addition of EPS from the bacterium *Alteromonas atlantica* and of commercially available xanthan gum, respectively, resulted in an increased erosion resistance of the sediment. These contradicting results may reflect differences in properties of bacterial EPS compared to the EPS produced by benthic diatoms. Extensive literature exists on the physico-chemical properties of bacterial EPS (Decho 1990; Sutherland 1990; Decho 2000) whereas little is known about the properties of diatom EPS. It has been suggested that mucilage produced by benthic diatoms either for the purpose of movement (Webster et al. 1985) or as a result of overflow metabolism (de Winder et al. 1999) easily dissolves in water. Its effect on sediment stability could be limited because this EPS would dissolve with every tide. On the other hand, Lewin (1956) showed that gel formation of EPS isolated from green algae occurred when divalent cations were added. Also, microscopic observations using low-temperature scanning electron microscopy (Yallop et al. 1994; Paterson 1995; Taylor & Paterson 1999) revealed a polymeric matrix between sediment grains in which EPS strands bound sediment particles together. In addition, the presence of EPS in amounts comparable to those used in this study, resulted in an increase in erosion threshold of natural intertidal sediments (chapter 6). Therefore, another possibility that must be considered is that the extraction procedure used to isolate and purify EPS from the sediment, irreversibly changed the tertiary structure preventing it to form a gel-like state. However, Dade et al (1990) used comparable extraction methods, including ultrafiltration and freeze drying, to isolate purified bacterial EPS. Their results showed a clear effect of the EPS on the properties of the sediment. Also, Moreno et al. (2000) used comparable methods to isolate EPS from the cyanobacterium *Anabaena* sp. Rheological measurements indicated that this EPS behaved as a weak gel.

Alternatively, it can be hypothesized that the action of the diatoms is necessary to structure the EPS in such a way that grain to grain adhesion is established. Decho (1994) emphasized that biofilms are heterogeneous, consisting typically of highly ordered and disordered regions. This suggests that the EPS matrix can be structured by the organisms themselves (see also Tolker-Nielsen & Molin 2000). Wang et al. (1997) reported that the EPS produced by *Achnanthes longipes* allowed these organisms to move and to attach to surfaces. It was observed that this EPS was highly structured. Disruption of the synthesis of these polysaccharides resulted in the inability to attach to substrata. Also, the polymers played a critical role in the development of colony morphology. Edgar & Pickett-Heaps (1984) suggested that diatoms secrete strands of EPS that attach to a surface, thereby facilitating movement through the sediment. In this way diatoms may physically alter sediment properties by binding sediment particles together via a "structured" type of EPS.

In summary, clear differences in sorption were observed between the two types of EPS (coll-SF and EDTA-SF) that were extracted from an intertidal mudflat. For both fractions sorption increased with increasing Ca^{2+}-concentrations indicating the importance of cation

bridging. Although the sorption of EPS shows the potential of EDTA-SF and to a lesser extend coll-SF to modify sediment properties in a way that can be observed in field situations, this was not observed by means of rheological measurements that were performed in this study. Therefore it is considered that other aspects than the presence of EPS itself (e.g. active structuring of the biofilm by the organisms present) are equally important when looking at mechanisms of biogenic stabilization in intertidal mudflats.

CHAPTER 10

GENERAL DISCUSSION

Excretion of extracellular carbohydrate by benthic epipelic diatoms

Benthic epipelic diatoms excrete a substantial portion of photosynhetically fixed carbon in the form of extracellular carbohydrate. Diatom cultures as well as natural populations of microphytobenthos excreted 40-75 % of fixed carbon into the environment (chapter 4, 5), confirming other estimates reported in literature (Goto et al. 1999; Middelburg et al. 2000; Smith & Underwood 2000). Transfer of fixed carbon to extracellular pools occurred over short time scales (within 30 min) and was proportional to the rate of photosynthesis, even under low light conditions (Chapter 4). These results indicate that carbohydrate excretion is important in benthic diatoms. It is therefore tempting to assume that this property provides a growth advantage that is essential for the survival of diatoms in intertidal mudflats (see also Ruddy et al. 1998b).

In order to study the production dynamics of EPS excretion by benthic diatoms, sequential extraction procedures were applied to isolate the EPS from cultures as well as sediment samples. In cultures, a fraction termed soluble EPS was isolated from the supernatant after centrifugation. Subsequently, the cell pellet was extracted in water (1 h at 30°C) which recovered a fraction that was termed bound EPS. In sediment samples, two EPS fractions (termed water- and EDTA-extractable EPS) were recovered by extraction of the sediment in water (1 h at 30°C) followed by extraction of the remaining cell pellet in 0.1 M EDTA (16 h, 20°C). The fractions that were obtained in this way were operationally defined. This means that the different EPS-fractions were only defined by their methods of extraction and did not have any relevance on forehand in relation to their mechanisms of excretion and their role(s) in intertidal mudflats. In order to come to an ecologically and physiologically relevant characterization of these operationally defined EPS fractions, detailed studies were performed examining the production dynamics, composition, localization and sorption characteristics of EPS obtained from culture or *in situ* experiments (chapter 2-5, 9). In this way, it was possible to obtain information on the roles of these EPS fractions and also to relate the production of EPS in cultures to the production in intertidal mudflats.

By applying these sequential extraction procedures it was shown that in axenic cultures of epipelic diatoms two types of EPS were excreted that differed to a large extend in terms of localization, composition and production dynamics (chapter 2, 3). Production of

bound EPS was dominant in the exponential growth phase and decreased during the course of growth, while the production of soluble EPS became dominant in the stationary phase. This indicates that the properties of the EPS that are excreted change during the course of growth and are dependent on the physiological status of the diatoms. The clear distinction between the two EPS-fractions suggests that they serve different functions and are under a different metabolic control. In literature, the physiological basis of the excretion of EPS by epipelic diatoms has been explained either by its role in motility (Smith and Underwood 1998, 2000) or as a result of overflow metabolism due to nutrient limitation (Bhosle et al. 1995; Staats et al. 2000b). The results presented in this thesis suggest that the excretion of bound EPS occurs as a result of overflow metabolism due to limited storage capacity for intracellular carbohydrates (Chapter 3), while the continuous release of soluble EPS in the stationary phase may be explained as motility related EPS excretion in response to stationary phase conditions (i.e. nutrient or light limitation). In this way EPS production would yield several profits: (1) The excretion of copious amounts of EPS enables diatoms to live in a biofilm mode of growth. The advantages for microorganisms to live in biofilms are numerous (Decho 1990; Neu 1994). For example, diatom biofilms effectively retain water in their matrix, which prevents benthic diatoms from desiccation. Furthermore, these biofilms increase the mud content of the sediment, which enhances the concentration of inorganic nutrients available to the diatoms (van de Koppel et al. 2001) (2) EPS-excretion maximizes the biosynthesis of storage carbohydrate in the light. These carbohydrates may be utilized in the dark and be used to sustain growth (chapter 2, 3). (3) EPS-excretion enables diatoms to migrate through the sediment in response to fluctuating conditions. Benthic diatoms migrate in response to tidal and diurnal cycles (Pinckney and Zingmark 1991; Serôdio et al. 1997) and may also move as a result of chemotaxis. Smith & Underwood (2000) suggested that production of soluble EPS was related to motility. In this thesis it was shown that production of this EPS occurred particularly during stationary phase (i.e. light or nutrient limitation), which confirms results of Ruddy et al. (1998a, b) who suggested that migration of diatoms occurred during periods of light or nutrient limitation in order to improve growth conditions.

In chapter 5, the *in situ* production dynamics of water- and EDTA-extractable carbohydrates were investigated with natural microphytobenthos over 24 h periods in order to determine short-term variations in extracellular carbohydrates associated with the microphytobenthos. In the presence of diatom biofilms, water-extractable carbohydrates behaved highly dynamic. In the light a tenfold increase was observed while this material disappeared during the subsequent immersion period. The chlorophyll *a*-normalized production of water-extractable EPS in the light was 4.5 h^{-1} (Table 10.1), which was in the same range compared to EPS production rates that were found in laboratory experiments (Table 10.1, chapter 3, 4). On the other hand, Underwood & Smith (1998b), estimated that chlorophyll *a*-normalized EPS-production in an intertidal mudflat during daytime emersion was 0.48 h^{-1}, which is an order of

Table 1: Maximum chlorophyll *a*-normalized EPS production rates in axenic diatom cultures and in microphytobenthos samples.

EPS-fraction	maximum EPS production rate (h^{-1})
soluble EPS (*C. closterium*)[a]	0.3
bound EPS (*C. closterium*)[a]	3.1
soluble EPS (*Nitzschia* sp.)[a]	0.1
bound EPS (*Nitzschia* sp.)[a]	1.8
water-extractable EPS (field sample)[b]	4.5
EDTA-extractable EPS (field sample)[b]	0.08
soluble EPS (*C. closterium,* ^{14}C-experiment)[a]	1.5
bound EPS (*C. closterium,* ^{14}C-experiment)[a]	4.1
soluble EPS (field sample, ^{14}C-experiment)[a]	6.9
bound EPS (field sample, ^{14}C-experiment)[a]	4.4
water-extractable EPS (Underwood & Smith 1998)[a]	0.48

a: EPS isolated by ethanol precipitation
b EPS isolated by ultrafiltration using a 100 kDalton filter

magnitude lower than the value reported above (Table 10.1). This may be explained by the differences in depth resolution of the sediment samples between the two studies. Underwood and Smith (1998b) measured EPS production in the upper 5 mm of the sediment while we used the upper 0.2 mm horizon. Paterson et al (2000) showed that diatoms were mainly active in the uppermost surface layer of the sediment. These authors also indicated that depth resolution was an influential factor in the study of EPS production. In contrast to the highly dynamic water-extractable carbohydrate fraction, EDTA-extractable carbohydrate behaved more conservative with a chlorophyll *a*-normalized production rate of 0.08 h^{-1}.

Water-extractable EPS showed clear resemblance with the bound EPS that were isolated from axenic diatom cultures (chapter 3), The production of bound as well as water-extractable EPS was strictly dependent on the light and both EPS fractions that were produced in the light contained a high relative amount of glucose. This suggests that the mechanism of EPS-production observed in the field was similar to that of the bound EPS in the cultures. This would mean that *in situ* EPS-production was a result of overflow metabolism rather than being a product of movement related EPS-excretion. In contrast, Smith & Underwood (1998) found that production of EPS occurred predominantly during periods of darkness. Our results did not show any dark production, which suggests that motility related EPS production was small compared to photosynthesis related production. These contradicting results may however be caused by differences in the physiological status of the microphytobenthic assemblages that were studied (see chapter 3).

The water-extractable EPS that were produced during daytime emersion contained 90 % glucose. At the start of the emersion period and deeper in the sediment the contribution of

glucose was considerably lower. This indicated that glucose was preferentially removed leaving a more biorefractory carbohydrate pool. The monosaccharide distributions of these biorefractory carbohydrates were comparable between extracellular carbohydrates isolated from geographically distant mudflats (chapter 8). Similar observations were made in the pelagic zone were the glucose portion of EPS excreted by phytoplankton was subject to rapid partial degradation (Alihuware & Repeta 1999). The remaining metabolically stable polysaccharides accumulated in surface seawater and were constant in composition over a wide range of geographical areas (Aluwihare et al. 1997). In addition, Stodegger & Herndl (1998) showed that bacteria utilized labile carbohydrates and converted a considerable fraction into more refractory extracellular material. Similar mechanisms may apply to intertidal benthic systems, were labile water-extractable carbohydrates, produced during daytime emersion, may quickly be converted into more refractory exopolysaccharides. In chapter 3 it was shown that labile bound EPS was utilized in the dark by benthic diatoms and converted to dissolved polysaccharides that had a more heterogeneous monosaccharide distribution. This indicated that benthic diatoms themselves might convert labile organic matter into more refractory exopolymers.

Extracellular carbohydrate distribution in intertidal mudflats

Spatial and temporal dynamics in the standing stocks of extracellular carbohydrates were examined over various temporal and spatial scales (chapter 6-8). Using non-parametric and parametric tests it was observed that a correlation existed between water-extractable carbohydrates and chlorophyll *a*. This was consistent with the predictive model proposed by Underwood & Smith (1998b). The relationship appeared to be valid over a range of intertidal mudflats in temperate areas (Chapter 8) and could be improved by taking into account different geomorphological features on a mudflat (Chapter 7). Underwood & Smith (1998b) hypothesized that this relation was the result of the production of EPS by benthic diatoms, while sediment characteristics would be of minor importance. This does not agree with the results presented in this thesis. Besides the relationship found between chlorophyll *a* and water-extractable carbohydrate, the water- and EDTA-extractable carbohydrate fractions also correlated with sediment grain size (chapter 6, 8). Sediment grain size may directly and indirectly influence the *in situ* carbohydrate content. The amount of organic matter adsorbed to sediment grains is inversely related with particle grain size (Bergamaschi et al. 1997). This is because finer sediment particles constitute a larger adsorptive area compared to coarser grains. Therefore, sediment grain size may largely determine the contents of extracellular carbohydrates when EPS-production is of minor importance (i.e. when microphytobenthos abundance is low, chapter 5). Furthermore, diatoms maintain higher growth rates on silt dominated sediments (van de Koppel et al. 2001), while diatoms themselves optimize growth

conditions by trapping fine-grained sediment in the biofilm (chapter 6). Therefore, a positive feedback exists between microphytobenthos and sediment grain size, which will lead to increased levels of extracellular carbohydrates. The relation between chlorophyll *a* and water-extractable carbohydrate is based on the measurement of standing stocks. In chapter 5 it was shown that contents of extracellular carbohydrates were highly variable over short time scales and considerable production of water-extractable carbohydrate was observed (chlorophyll *a*-normalized EPS-production rate of 4.5 h^{-1}, table 10.1). Underwood & Smith (1998b) calculated a ratio of *in situ* water-extractable EPS : chlorophyll *a* of 2.62 that was similar for a variety of different mudflats. In contrast, we calculated a ratio of 30 at the end of a daytime emersion period (chapter 5), which shows that the model of Underwood & Smith (1998b) does not account for the considerable amount of water-extractable carbohydrate that is excreted during daytime. Considering these facts it seems unlikely that the correlation can solely be interpreted in terms of the production by microphytobenthos. More likely, the factors that determine carbohydrate contents in the sediment are multiple and the correlation between chlorophyll *a* and water-extractable carbohydrate content reflects the result of a complex interaction of biological, physical and sedimentological processes. In chapter 8 it was shown that dynamics in the contents of extracellular carbohydrates took place over larger areas, typically the scale of mudflats. Hydrodynamic processes like tidal currents and wave action typically have an effect on a comparable spatial scale. In addition, the resuspension of microphytobenthos and sediment transport processes are governed by wind generated waves, while these variables are also correlated with the content of extracellular carbohydrate. Therefore, it seems likely that the distribution of extracellular carbohydrates in intertidal mudflats is at least to some extent determined by the hydrodynamic regime acting upon these areas.

The role of benthic diatoms in the morphodynamics of intertidal mudflats

From chapter 6 it was clear that biostabilization of intertidal sediments was associated with the presence of diatom biofilms that were visible at the sediment surface. This coincided with high contents of chlorophyll *a* and extracellular carbohydrates. It has been suggested that diatom biofilms have little effect on sediment properties because diatoms generally occur in areas that are already characterized by a high mud content and low hydrodynamic forcing (Staats et al. 2001). However, our results demonstrated that the presence of diatom biofilms had a large impact on the dynamics and characteristics of the sediment, which was reflected in the patterns in sediment stability, morphology, bed level and grain size distribution (chapter 6). This process of biogenic stabilization was temporal but specifically associated with the presence of diatom biofilms (see also Kornman & de Deckere 1998). The disappearance of these biofilms was rapid and probably caused by the increased abundance of meio- and

macrofauna in combination with the prevailing hydrodynamic regime. In the absence of diatom biofilms, sediment fluxes were characterized by net offshore sediment transport generated by wind dominated hydrodynamic conditions (see also Christie et al. 1999). Hence, under conditions that favor diatom growth (i.e. low grazing pressure, low hydrodynamic forcing, sufficient nutrient and light levels) sediment stabilizing biofilm structures are rapidly formed whereas under conditions that are detrimental for diatom growth sediment processes appear to be governed largely by the hydrodynamics acting upon a mudflat.

In the Biezelingse Ham mudflat, sediment transport events were derived from monthly changes in bed level. Clearly, the presence of diatom biofilms resulted in net sediment deposition, while in the absence of diatom biofilms sediment erosion was observed. The total variation in bed level varied between 2.5 and 6 cm, which is within the range of estimates reported by other workers (Frostick & McCave 1979; O'Brien et al. 2000; Herman et al. 2001). Frostick & McCave (1979) postulated that that in the Deben estuary (United Kingdom), on a seasonal basis up to 10^5 tons of sediment (calculated on the basis of 5 cm accretion) were transferred between the channels and the mudflats. These authors suggested that sediment accretion was the result of the activity of benthic diatoms. Furthermore, Herman et al. (2001) calculated that in the Westerschelde estuary (The Netherlands) a seasonal deposition of 5 cm of sediment covering 40 % of the intertidal flats in this estuary would largely explain seasonal fluctuations in suspended sediment concentrations in the water column. These results also indicate that a seasonal deposition mediated by benthic diatoms may be a significant factor determining the sediment balance of estuaries.

A tight coupling between biology, sedimentology and physical processes was observed when geomorphological ridge-runnel features were investigated in the Humber mudflat (chapter 7). The mechanism of formation of these ridge-runnel features is hitherto unknown at the moment but it was suggested that they might appear as the result of specific hydrodynamic conditions (Dyer 1998; O'Brien et al. 2000). In the ridges, chlorophyll *a* was positively correlated with water-extractable carbohydrate contents while this was not the case in the runnels. This may be explained by the fact that the ridges were completely emerged during low tide. Therefore accumulation and subsequent turnover of extracellular carbohydrate could occur, leading to the observed relation between chlorophyll *a* and water-extractable carbohydrates. In contrast, runnels always contained a layer of water, which probably resulted in a quick dissolution of produced carbohydrates, preventing their accumulation in the sediment. In addition, a higher bulk density was reported in the ridges compared to the runnels (Christie et al. 2000), which was attributed to the differences in flooding regime during low tide. Both the distinction in sedimentological and biological properties between ridges and runnels suggest a difference in sediment stability. Indeed, Paterson et al. (2000) observed that sediment stability was higher in the ridges compared to the runnels. This was reflected in the higher erosion threshold and the lower relative erosion

rate in the ridges. These results suggest that ridge-runnel features are self sustaining due to the combined action of sedimentology (bulk density) and biology (algal carbohydrate excretion), (see also Amos et al. 1998; Christie et al. 2000), resulting in stable ridges and less stable runnels.

It is well established that the stabilization of intertidal sediments by benthic diatoms can be the result of the excretion of EPS by these organisms. The role of EPS in the process of biogenic sediment stabilization has been inferred from correlative studies (Kornman & de Deckere 1998; Sutherland et al. 1998; Paterson et al. 2000). Although in these studies a correlation between EPS-content and sediment stability was reported, it is the effect of the diatom biofilm as a whole that is described. In chapter 9, water- and EDTA-extractable EPS were isolated from intertidal sediments and added to sediment slurries in order to study the adsorption of EPS and its effect on the sediment properties. In this experiment, addition of neither the water- nor the EDTA-extractable fraction altered the rheology of the sediment slurries. This indicated that other factors than the presence of EPS alone play a role in the stabilization of sediments. It was hypothesized that the diatoms themselves structure the EPS matrix in such a way that grain to grain adhesion is established (chapter 9). This appears to be valid in muddy sediments were increases in sediment stability go together with increases in both chlorophyll *a* and extracellular carbohydrate levels (Sutherland et al. 1998; Kornman & de Deckere 1998; chapter 6). However, in the sandy station in the Biezelingse Ham (chapter 6) this pattern was not evident. In this station stability was correlated with EDTA-extractable carbohydrate while chlorophyll *a* contents were consistently low. Therefore, mechanisms of sediment stabilization in this sandy station may be different compared to muddy sediments. Although the addition of EPS did not affect sediment properties, the water- and EDTA-extractable EPS fractions differed in their adsorptive characteristics. Little water-extractable EPS adsorbed to sediment particles compared to EDTA-extractable EPS. This suggests that water-extractable EPS is not very effective in the process of sediment adhesion. Hence, its role in sediment stabilization may be limited. Underwood & Smith (1998b) and de Winder et al. (1999) hinted to the possibility that water-extractable carbohydrates were rapidly dissolved into the overlying water during tidal immersion. In addition, results presented in chapter 5 indicate that water-extractable carbohydrates represent a dynamic and labile fraction, which may not be of great importance for sediment stabilization. In flume experiments, Sutherland et al. (1998) observed that contents of water extractable carbohydrates stayed consistently low when *Nitzschia curvilineata* formed sediment-stabilizing biofilms. These results strongly suggests that exopolymers that have a higher degree of association with the sediment (e.g. EDTA-extractable carbohydrate) may be more important determining sediment stabilization whereas the importance of water-extractable carbohydrate is predominantly in its role as a labile carbohydrate source for heterotrophic consumers.

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SUMMARY

Intertidal mudflats are highly dynamic systems that are characterized by rapid changes in environmental variables. Notwithstanding the rapidly fluctuating conditions, intertidal mudflats represent highly productive ecosystems in which epipelic diatoms are the dominant primary producers. These are unicellular algae with a silica frustule enveloping the cell. Epipelic diatoms are able to move through the sediment in response to the light and the tidal cycle. An important property of these organisms is that they excrete a substantial amount of photosynthetically fixed carbon as extracellular polymeric substances (EPS) that mainly consist of polysaccharides. These EPS form a matrix in which sediment particles and cells are embedded such that the diatoms develop as a biofilm that protects them from the rapidly changing conditions in mudflats. Diatom biofilms increase the erosion threshold of the sediment and thereby they may have an effect on the morphological development of intertidal areas.

Two main research questions were formulated in this thesis. (1) What are the mechanisms and relevant scales of EPS production by benthic diatoms? (2) What is the role of the production of extracellular carbohydrates by benthic diatoms in the morphological development of intertidal mudflats? In order to answer these questions, production dynamics of extracellular carbohydrates were investigated in cultures of benthic diatoms and detailed surveys were undertaken in a number of intertidal mudflats.

In chapter 2 and 3 production dynamics of EPS in axenic cultures of benthic diatoms were investigated under alternating light – dark conditions. It was shown that diatoms excreted two distinct types of EPS that differed in terms of localization, production dynamics and composition. One type of EPS (termed soluble EPS) was produced continuously once cultures entered the stationary phase. Soluble EPS was rich in uronic acids and galactose. It was suggested that the production of soluble EPS was related to the ability of diatoms to migrate in response to non-optimal circumstances such as adverse light or nutrient conditions. In contrast, production of bound EPS occurred exclusively in the light and the glucose-rich polymers that were produced were to a large extent degraded during the subsequent dark period. The diatom cultures were bacteria free and therefore it was concluded that the diatoms themselves utilized the bound EPS in the dark. Bound EPS resembled intracellular storage carbohydrates in terms of production dynamics and monosaccharide distribution (chapter 3). This suggested that bound EPS were produced as a result of overflow metabolism due to limited capacity of benthic diatoms to store carbohydrates internally. The production of extracellular carbohydrate was proportional to the rate of photosynthesis and a substantial part of photosynthetically fixed carbon (40-75 %) was excreted within a period of 30 minutes

(chapter 4). This indicated that fluctuations in extracellular carbohydrates may occur within short time periods. It was concluded that short time scales (minutes - hours) must be considered when studying EPS excretion by benthic diatoms.

Sequential extraction procedures were also applied for the study of extracellular carbohydrates in intertidal mudflats. *In situ* studies over 24 h periods (chapter 5) showed that water-extractable carbohydrates represented a highly dynamic fraction. In contrast, EDTA-extractable carbohydrates behaved more conservative. Water-extractable carbohydrates were produced in the light only and consisted of polysaccharides that were rich in glucose. The dynamics and composition of the water-extractable carbohydrate fraction were similar to those of the bound EPS in the culture experiments. This indicated that the *in situ* production of extracellular carbohydrate was the result over overflow metabolism rather then being a product of diatom movement. The EDTA-extractable carbohydrate fraction represented more refractory carbohydrates that probably originated as a result of partial degradation and conversion of labile carbohydrate by heterotrophic consumers. This may include the heterotrophic utilization of labile carbohydrate by diatoms themselves and the subsequent conversion into more refractory motility-related exopolysaccharides (chapter 3).

Standing stocks of water-extractable carbohydrates were correlated with diatom biomass (measured as chlorophyll *a*) and with sediment grain size (chapter 6, 8). This strongly suggested that both biological and sedimentological processes determined the carbohydrate contents in intertidal sediments. In addition, hydrodynamic processes such as tidal currents and wind generated waves play a role in transport of sediment particles and the resuspension of microphytobenthos. Therefore, the hydrodynamic regime acting upon a mudflat may indirectly influence sediment carbohydrate contents. Hence, the content of water-extractable carbohydrates in intertidal sediments is probably not solely a result of carbohydrate production by microphytobenthos but is determined by a complex interaction of biological, sedimentological and physical processes.

In a similar way, the morphodynamics of intertidal mudflats were determined by a complex interplay of biotic and abiotic processes (chapter 6, 7). In chapter 6 it was shown that diatom biofilms had a strong temporal effect on the morphology of an intertidal mudflat. When diatom biofilms were present, the sediment stability increased and fine-grained sediment particles were trapped in the biofilm matrix causing net deposition of sediment on top of the mudflat. Furthermore, diatom biofilms effectively prevented sediment erosion during periods of high hydrodynamic forcing. In late spring, diatom biofilms quickly disappeared, which was probably the result of grazing by meio-and macrofauna in combination with wind generated waves. In the absence of diatom biofilms sediment stability was low and sediment erosion occurred. During this period mudflat morphology appeared to be determined mainly by hydrodynamic processes.

Examination of specific bedforms in the Humber mudflat (United Kingdom) also revealed a close coupling between biological and physical processes (chapter 7). The Humber mudflat is characterized by the presence of extensive ridge - runnel structures, which are parallel corrugations that are situated in a shore normal position. The ridges were emerged during low tide and were therefore subject to drying. The runnels are drainage-like structures, which permanently contained water. On the ridges, a positive correlation was observed between water-extractable carbohydrates and diatom biomass. In the runnels such a correlation was not observed. Furthermore, bulk density was lower in the runnels compared to the ridges, while sediment stability was higher on the ridges compared to runnels. This suggested that ridge - runnel features are self-sustaining as a result of the combined action of sedimentology (bulk density) and biology (algal carbohydrate excretion).

It has been argued that biogenic stabilization of intertidal mudflats is attributed to the action of exopolysaccharides, which bind sediment particles together thereby forming a stabilized surface layer. The effect of EPS alone on the sedimentological properties was investigated by the addition of isolated preparations of water- or EDTA-extractable EPS to sediment slurries (chapter 9). Neither the water- nor the EDTA-extractable EPS had any effect on the sediment properties as was measured by the rheology of the sediment slurries. Because *in situ* EPS-concentrations comparable to those used in the addition experiments clearly changed the properties of the sediment, it was suggested that the diatoms themselves may structure the EPS in such a way that grain to grain adhesion is established. Although neither of the EPS fractions affected sediment properties, the water- and EDTA-extractable carbohydrate fractions clearly differed in their sorption to the sediment. Sorption of water-extractable EPS to sediment particles was low compared to EDTA-extractable EPS. This suggested that water-extractable EPS was not very effective in the process of sediment adhesion. Rather, this EPS fraction is important as a labile carbohydrate source for heterotrophic consumers. Because of its higher degree of association with sediment particles EDTA-extractable EPS may potentially be more important in the process of biogenic stabilization by benthic diatoms.

SAMENVATTING

Getijdenplaten worden gekarakteriseerd door snelle veranderingen in omgevingsvariabelen. Ondanks de hoge dynamiek zijn getijdenplaten zeer productieve ecosystemen waarin epipelische diatomeeën in belangrijke mate verantwoordelijk zijn voor de primaire productie. Deze diatomeeën zijn eencellige algen waarvan de cel omgeven wordt door een silicaatskelet. Epipelische diatomeeën bewegen door het sediment in reactie op onder andere de heersende licht condities en het getij. Een belangrijk kenmerk van deze organismen is dat ze een substantieel deel van het fotosynthetisch gefixeerd koolstof uitscheiden als Extracellulaire Polymere Substanties (EPS) die voornamelijk bestaan uit polysacchariden. Dit EPS vormt een matrix waarin sediment deeltjes en cellen verankerd worden. Hierdoor worden de diatomeeën beschermd tegen de snel fluctuerende omstandigheden op getijdenplaten. Tevens verhogen diatomeeën biofilms de stabiliteit van het sediment oppervlak waardoor ze mogelijk invloed uitoefenen op de morfologische ontwikkeling van getijdengebieden.

In dit proefschrift stonden twee onderzoeksvragen centraal: (1) Wat zijn de mechanismen van EPS productie door benthische diatomeeën en wat zijn de relevante tijd- en ruimteschalen waarbinnen EPS productie zich afspeelt? (2) wat is de invloed van de uitscheiding van extracellulaire koolhydraten door benthische diatomeeën op de morfologische ontwikkeling van getijdenplaten. Om deze vragen te beantwoorden werden studies verricht naar de productie dynamiek van extracellulaire suikers in axenische cultures van benthische diatomeeën en in een aantal verschillende getijdengebieden.

In hoofdstuk 2 en 3 werd de productie dynamiek van EPS onder wisselende licht - donker condities bestudeerd in axenische diatomeeën cultures. Aangetoond werd dat de diatomeeën twee soorten EPS produceerden die duidelijk verschillend waren in productie dynamiek, lokalisatie en samenstelling. EPS verkregen door centrifugeren van de culture, opgelost EPS genoemd, werd continue geproduceerd op het moment dat de cultures overgingen in de stationaire fase. Dit opgelost EPS bevatte hoge relatieve hoeveelheden aan uronzuren en galactose. De eigenschappen van het opgeloste EPS wijzen op een mogelijke rol bij de voortbeweging van diatomeeën in reactie op condities van licht- of nutriënt limitatie. De andere EPS fractie, gebonden EPS genoemd, werd verkregen door extractie in water bij 30°C. Gebonden EPS werd uitsluitend geproduceerd in het licht. De geproduceerde polymeren waren rijk in glucose en werden grotendeels afgebroken gedurende de opeenvolgende donker periode. De diatomeeën cultures waren vrij van bacteriën en aan de hand daarvan werd geconcludeerd dat het gebonden EPS door de diatomeeën zelf benut werd gedurende de donker periode. Zowel de productie dynamiek als de monomere suikersamenstelling van het gebonden EPS vertoonde duidelijke overeenkomsten met die van

intracellulaire reserve koolhydraten. Dit duidde erop dat gebonden EPS werd uitgescheiden als gevolg van overflow metabolisme door een beperkte capaciteit van diatomeeën om koolhydraten binnen de cel op te slaan. De productie van extracellulaire koolhydraten was evenredig gerelateerd met de snelheid van fotosynthese en een aanzienlijk deel van de gefixeerde koolstof (40-75 %) werd binnen 30 minuten uitgescheiden. Dit gaf aan dat fluctuaties in extracellulaire koolhydraten kunnen optreden binnen een kort tijdsbestek. Deze korte tijdschalen (minuten-uren) moeten in acht genomen worden wanneer de mechanismen van EPS uitscheiding door diatomeeën bestudeerd worden.

Voor het onderzoek naar extracellulaire koolhydraten in de getijdenplaten werd eveneens gebruik gemaakt van sequentiële extractiemethoden. 24 Uur experimenten in een getijdenplaat toonden aan dat de water-extraheerbare suiker fractie een zeer dynamische fractie vertegenwoordigde. De EDTA-extraheerbare suiker fractie was meer conservatief. De productie van water extraheerbare koolhydraten vond alleen plaats in het licht en de gevormde polysacchariden bestonden grotendeels uit glucose. Zowel de samenstelling als de dynamiek van water extraheerbare koolhydraten vertoonden sterke gelijkenis met de gebonden EPS fractie in de cultuur experimenten. Dit duidde erop dat de productie van extracellulair koolhydraat in de getijdenplaat eerder een gevolg was van overflow metabolisme dan dat er sprake was van uitscheiding ten behoeve van de beweging van diatomeeën. EDTA extraheerbare koolhydraten representeerden een meer stabiele fractie die waarschijnlijk gedeeltelijk ontstond door partiele degradatie en omzetting van labiele suikers door heterotrofe organismen. Voor benthische diatomeeën werd in het donker de omzetting aangetoond van labiele polysacchariden naar meer stabiele (aan beweging gerelateerde) polymere suikers (hoofdstuk 3).

De gehaltes aan water extraheerbare suikers aanwezig in getijden sedimenten waren gecorreleerd met diatomeeën biomassa (gemeten als chlorophyll *a*) alsmede met de korrelgrootte van het sediment (hoofdstuk 6, 8). Dit duidde erop dat zowel biologische als sedimentologische processen de koolhydraat gehaltes in getijdenplaten bepaalden. Verder zijn hydrodynamische processen als getijdenstromen en wind gegenereerde golven belangrijk voor het transport van sediment deeltjes en de resuspensie van microphytobenthos. Dit betekent dat het hydrodynamisch regime boven een getijdenplaat een indirecte invloed heeft op het koolhydraat gehalte in het sediment. Het is daarom aan te nemen dat het gehalte aan water extraheerbare suikers eerder het resultaat is van een complexe interactie tussen biologische sedimentologische en fysische processen dan dat het alleen bepaald wordt door de productie van extracellulaire suikers door microphytobenthos.

Op een zelfde manier werden veranderingen in de morfologie van getijdenplaten bepaald door een complex samenspel van biotische en abiotische processen (hoofdstuk 6, 7). De resultaten uit hoofdstuk 6 maakten duidelijk dat diatomeeën biofilmen een sterk tijdelijk effect hadden op de morfologie van getijdenplaten. De aanwezigheid van deze biofilmen

resulteerde in een verhoogde stabiliteit van het sediment oppervlak. Verder werden slib deeltjes ingevangen in de biofilm matrix resulterend in een netto depositie van sediment op de getijdenplaat. Tevens bleek dat ook bij hoge wind snelheden (onder condities waarbij in de afwezigheid van biofilms aanzienlijke sediment erosie optreedt) diatomeeën biofilmen effectief waren in het voorkomenvan sediment erosie. De diatomeeën biofilmen verdwenen in snel tempo aan het einde van de lente, wat waarschijnlijk werd veroorzaakt door begrazing door meio- en macrofauna in combinatie met een hoge wind activiteit. De sediment stabiliteit was laag in de afwezigheid van diatomeeën biofilmen, wat resulteerde in netto sediment erosie. Gedurende deze periode leek de morfologie van de getijdenplaat voornamelijk bepaald te worden door hydrodynamische processen.

In een getijdenplaat in het Humber estuarium (Groot-Brittannië) werd een studie verricht naar specifieke sediment structuren die zichtbaar waren op het sediment oppervlak. Uit deze studie bleek eveneens dat er een sterke koppeling bestond tussen biologische en fysische processen (hoofdstuk 7). De getijdenplaat in het Humber estuarium werd gekarakteriseerd door de aanwezigheid van uitgebreide ridge-runnel structuren. Dit zijn structuren van parallel lopende ribbels die ongeveer loodrecht gepositioneerd zijn ten opzicht van de kustlijn. De toppen van deze structuren (ridges) vielen droog gedurende de laag water periode en waren daardoor onderhevig aan uitdroging. De dalen (runnels) bleven ook tijdens de laag water periode gevuld met een hoeveelheid water. In de ridges werd een positieve correlatie gevonden tussen chlorophyll *a* en de water extraheerbare suiker fractie. Dit was echter niet het geval in de runnels. Verder was de dichtheid van het sediment lager in de runnels vergeleken met de ridges, terwijl een hogere sediment stabiliteit werd gemeten in de ridges. Dit duidde erop dat de combinatie van sedimentologische (dichtheid) en biologische processen (uitscheiding van koolhydraten door microphytobenthos) een rol speelden bij de instandhouding van deze specifieke sediment structuren.

De biologische stabilisatie van getijdensedimenten wordt vaak toegeschreven aan de activiteit van EPS. Vermoed wordt dat EPS functioneert als een lijm waardoor sediment deeltje gebonden worden wat uiteindelijk resulteert in een gestabiliseerde oppervlakte laag. Het effect van geïsoleerde preparaten van water extraheerbaar of EDTA-extraheerbaar EPS op het gedrag van het sediment werd onderzocht door toevoeging van deze typen EPS aan sediment slurries. Analyse van de rheologie van de sediment slurries gaf aan dat geen van de beide typen EPS de eigenschappen van het sediment veranderden. Vergelijkbare EPS gehaltes *in situ* hadden een duidelijk effect op de eigenschappen van getijdensedimenten. Dit suggereert dat het EPS in gestructureerde vorm wordt uitgescheiden door de diatomeeën waardoor adhesie tussen sediment deeltjes plaatsvindt. Hoewel de EPS addities de sediment eigenschappen niet beïnvloedden was er een duidelijk verschil in sorptie van de water extraheerbare en de EDTA-extraheerbare EPS fracties aan het sediment. Sorptie van water extraheerbaar EPS was laag vergeleken met adsorptie van EDTA-extraheerbaar EPS. Dit

suggereert dat water extraheerbaar EPS niet zeer effectief is voor sediment adhesie. Deze fractie lijkt daarom voornamelijk belangrijk als een bron van labiele koolhydraten voor heterotrofe organismen. Door de hogere mate van associatie met sediment deeltjes speelt EDTA-extraheerbaar EPS potentieel een belangrijkere rol in het proces van biologische stabilisatie in getijdengebieden.

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CURRICULUM VITAE

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