J.F.C. de Brouwer, T.R. Neu and L.J. Stal

On the function of secretion of extracellular polymeric substances by benthic diatoms and their role in intertidal mudflats: A review of recent insights and views

Abstract

Benthic diatoms produce large amounts of extracellular polymeric substances (EPS). These EPS's are believed to play important roles in physiological as wel as ecological processes in intertidal environments. Nevertheless, the function of these EPS's and the role they play in mediating physical and biological processes are currently poorly understood. This paper attempts to review current insight and views on this topic. The focus is on the complexity of EPS, which reveals itself at different levels. It has become clear that diatoms secrete different types of EPS. Moreover, the composition of EPS may vary among different species. These variations lead to the formation of different but distinct and ordered tertiary EPS structures as was shown by confocal laser scanning microscopy of diatom aggregates. These structural aspects of EPS secreted by benthic diatoms should be taken into account in any research that aims at explaining its role in the ecology of intertidal systems.

Introduction

Intertidal mudflats are highly dynamic systems that are characterized by rapid changes in environmental variables. Both diurnal light- and tidal cycles impact mudflats, inducing rapid fluctuations in light, salinity, temperature, water content, oxygen, and other environmental parameters. Under submerged conditions, sediment particles are transported (eroded from the surface or deposited on top of the mudflat) caused by physical processes such as wave action and tidal currents. Hence, the sediment-water interface is a highly dynamic environment.

Notwithstanding these dynamic conditions, intertidal mudflats are highly productive systems in which epipelic diatoms are the most important primary producers (Underwood and Kromkamp 1999). They supply the organic matter for the benthic (Middelburg *et al.* 2000) and planktonic foodwebs (De Jonge and Van Beusekom 1992), and could provide up to 50 % of total primary production in estuaries (Underwood and Kromkamp 1999). Epipelic diatoms secrete a considerable part of the photosynthetically fixed carbon as Extracellular Polymeric Substances (EPS) that

consist for a large part of carbohydrates (Hoagland *et al.* 1993; Staats *et al.* 1999). Because of the copious secretion of EPS, diatoms become embedded in a matrix of these polymers which are attached to the sediment. These structures are known as diatom mats or biofilms and are common in intertidal mudflats. By living in a biofilm, epipelic diatoms create their own stable microenvironment that allows them to cope with the rapidly changing conditions in intertidal mudflats (Decho 1994). Moreover, diatom biofilms enhance the stability of the sediment surface by increasing the erosion threshold (Paterson 1997, and references therein, Kornman and De Deckere 1998). Hence, diatoms have profound effects on the morphodynamics of mudflats (Dyer 1998) and influence sediment transport, potentially over the scale of whole estuaries (Frostick and Mccave 1979).

In spite of the importance of EPS production by benthic diatoms, the factors that control this process as well as its function are poorly understood. The complex interactions that take place between the diatoms and the biological and physical environment they live in make its study difficult. Therefore an important part of the research has been carried out on laboratory cultures of isolated strains of benthic diatoms. While it is evidently much easier to study the ecophysiology of EPS-production under controlled conditions in pure cultures, it is a strong drawback that the organisms are removed from their natural environment. However, the combination of these two approaches may give clues that lead to the elucidation of the role of EPS both for the diatoms and the biofilm intrinsically as well as for intertidal mudflats as a whole and even at the scale of the estuarine ecosystem. This paper attempts to review current insights and views on the possible functions of EPS secreted by benthic diatoms in intertidal areas.

Methodological considerations of the study of EPS

The most common method to investigate EPS production by benthic diatoms is based on its isolation from a sample matrix. However, a universal method for the extraction of EPS does not exist and different investigators have developed different protocols for the isolation of EPS from cultures (Smith and Underwood 2000; Staats *et al.* 1999; Wustman *et al.* 1998) or from sediment samples (Chiovitti *et al.* 2003b; De Brouwer *et al.* 2000; Underwood and Paterson 2003; Underwood *et al.* 1995). Analysis of these fractions has mainly focused on the carbohydrate part of the EPS. Although carbohydrates often represent a major part of the EPS secreted by diatoms (Bhosle *et al.* 1995; Staats *et al.* 1999), it should be kept in mind that other components such as proteins, lipids and nucleic acids may form part of the EPS matrix (Lawrence *et al.* 2003). EPS-fractions that are recovered by any extraction are principally operationally defined and give *a priori* no information about their characteristics, their mechanisms of secretion or their possible functions. Moreover, it is largely unknown to what extend these operationally defined extracellular fractions contain contaminations originating from intracellular compounds. In diatom cultures, isolation of EPS from the culture supernatant has become an accepted method yielding a fraction that is sometimes referred to as 'colloidal EPS' (Smith and Underwood 2000)

or as 'soluble EPS' (De Brouwer *et al.* 2002b). Centrifugation as a procedure to separate the cells and their extracellular polymeric compounds would likely avoid contamination by intracellular material (Staats *et al.* 1999), but it also results in incomplete recovery of EPS (De Brouwer *et al.* 2002b; Wustman *et al.* 1997). In order to also collect this more tightly bound EPS, sequential extraction protocols have been developed. However, more severe extraction procedures may damage the cells leading to contamination of the EPS by intracellular compounds. Hence, any protocol must be accompanied by rigorous controls and can not be carelessly employed for other organisms. Only few studies have discussed these problems in detail (Nielsen and Jahn 1999). Wustman *et al.* (1997) extracted EPS from different stalk forming diatoms and followed the extraction procedure by using differential interference contrast microscopy (DIC) while visualizing the EPS with alcian blue. They showed that extraction of the diatom *Achnanthes longipes* using water at 90 °C removed the bulk of the intracellular material while the extracellular stalks remained intact. These stalks were subsequently removed by hot bicarbonate extraction at 95 °C. These authors also showed that stalks of the freshwater diatom *Cymbella cistula* were removed by a treatment at 23 °C in 0.2 M EDTA and *trans*-1,2-diaminocyclohexane-N,N,N,',N'-tetraacetic acid but this procedure did not remove the stalks of *A. longipes*. This indicates that the characteristics of the EPS vary among diatom species emphasizing that extraction protocols must be evaluated for each specific case. There are various methods that give clues about possible contamination by intracellular compounds. For instance, Staats *et al.* (1999) monitored the amount of protein in fractions that were extracted by warm (30 °C) water, reasoning that a compromised cell membrane would leak and proteins are readily water soluble. De Brouwer *et al.* (2002b) used the fluorescent marker DIBAC (bis-(1,3-dibarbituric acid) which enters cells with a compromised cell membrane, in order to monitor the cell integrity of *C. closterium* under different extraction protocols. Chiovitti *et al.* (2004) showed that the glucose rich material that was extracted using warm (30 °C) water consisted of a β-1,3-glucan, which lead them to conclude that it represented the internal storage glucan chrysolaminaran rather than EPS. This view is subject of debate (Chiovitti *et al.* 2004; De Brouwer and Stal 2004), but it shows the importance to identify the nature of these operationally defined fractions.

Extraction of EPS from sediment samples is even more complicated compared to the extraction of cultured cells. One of the main reasons for this is that sediments represent ill-defined biological matrices that have a wide range of interactions with the sedimentary environment. Sediment particles are coated with organic matter. The amount of organic matter adsorbed to sediment particles is inversely related to grain size (Bergamaschi *et al.* 1997). This is because finer sediment particles constitute a larger adsorptive area compared to coarser grains. Subsets of extracted organic matter, commonly analyzed as carbohydrate (Underwood *et al.* 1995), show a similar inverse relationship with grain size (De Brouwer *et al.* 2000; De Brouwer *et al.* 2003; Paterson *et al.* 2000). Therefore, the presence of carbohydrate does not only depend on phototrophic biomass (Underwood and Smith 1998) but also on sediment characteristics. This is especially the case when EPS production is low because of a low abundance of microphytobenthos. Additionally, intertidal sediments are inhabited by

other organisms than microphytobenthos and these may also produce EPS. Such organisms include macrofauna (Meadows *et al.* 1990), meiofauna (Riemann and Schrage 1978) and bacteria (Dade *et al.* 1990). Although, current knowledge suggests that benthic diatoms are the major producers of EPS in intertidal mudflats (Underwood and Paterson 2003), the involvement of other organisms in the production, modification and degradation of EPS is currently largely unknown.

Another complicating factor in the extraction of EPS from sediments is related to sample handling. Sediment samples are often lyophilized prior to the extraction of EPS. The advantage of lyophilized samples is that water is removed creating a uniform sample matrix. However, several studies have shown that lyophilization causes lyses of the diatoms through which intracellular carbohydrates may be co-extracted (De Brouwer *et al.* 2000; Wigglesworth-Cooksey *et al.* 2001). Hence, the correlation between chlorophyll *a* and carbohydrate which is often reported for intertidal mudflats (Blanchard *et al.* 2000; Underwood and Smith 1998) is possibly in part attributed to intracellular compounds.

Although the extraction procedures for EPS from cultures of diatoms and field samples are easy to apply and may seem straightforward, the interpretation of the results remains difficult. There is an urgent need for a more comprehensive approach that allows tracing back the extracted material to its source. There is probably little to gain by optimizing the extraction procedures, hence our efforts should concentrate on the analytical side of the extracted material. A comprehensive analysis should give information about the origin (i.e. the organisms that produced it or diagenetically altered sedimentary organic matter) and allow the distinction between intra- and extracellular material. This may be done by using preparative techniques such as size fractionation (De Brouwer and Stal 2001; Wustman *et al.* 1998) or electrophoresis (Chiovitti *et al.* 2003a; Puskaric and Mortain-Bertrand 2003). In addition, microscope techniques can be used for exopolymer characterization or to monitor the extraction of EPS from diatoms.

By using Atomic Force Microscopy Higgins *et al.* (2002) identified two different types of EPS that differed in mechanical properties. The same technique was used to follow the extraction of EPS from *Pinnularia viridus* cells (Chiovitti *et al.* 2003b; Higgins *et al.* 2002). These authors showed that the differences in composition of the different carbohydrate fractions were associated with changes in the morphology and properties of the cell surface mucilage. Furthermore, fluorescence microscopy and confocal laser scanning microscopy have been used in combination with the application of fluorescently labeled lectins to localize EPS structures in isolated diatoms (De Brouwer *et al.* 2005; Neu 2000; Wustman *et al.* 1997) or natural phototrophic assemblages (Neu 2000; Norton *et al.* 1998). Although lectins recognize specific carbohydrate sequences in the EPS, it is not straightforward to distinguish between species specific EPS. By using antibodies raised against species specific EPS fractions it has been possible to analyze and localize the distribution of specific intracellular (Chiovitti *et al.* 2004) and extracellular glycoconjugates (Kawaguchi *et al.* 2003; Lind *et al.* 1997; Wustman *et al.* 1998). Furthermore, application of antibodies identified the role of extracellular glycoproteins in gliding movement and substratum adhesion (Lind *et al.* 1997).

Composition of EPS

In addition to quantitative information about exopolymers secreted by the diatoms, knowledge about the chemical composition may provide additional clues to understand the mechanism(s) of EPS-excretion and their function(s) in intertidal sediments. Generally, diatom-derived EPS-fractions are dominated by carbohydrates, the remaining part being mainly proteins and sulfate-groups (Bhosle *et al.* 1995; Hoagland *et al.* 1993; Staats *et al.* 1999; Wustman *et al.* 1997). Therefore, the majority of studies investigating the structure of EPS have focused on the analysis of carbohydrates originating from cultures of benthic diatoms (Allan *et al.* 1972; Chiovitti *et al.* 2003b; De Brouwer and Stal 2002; Staats *et al.* 1999; Underwood *et al.* 2004; Wustman *et al.* 1997) or from intertidal sediment samples (Cowie and Hedges 1984; De Brouwer *et al.* 2003; De Brouwer and Stal 2001; De Winder *et al.* 1999; Taylor *et al.* 1999). Meta-analyses of monosaccharide distributions of EPS-fractions originating from different species of benthic diatoms or from intertidal sediments indicated that considerable differences exist in the composition of EPS (Underwood and Paterson 2003). Cluster analysis identified two major EPS-types that could further be distinguished into 6 groupings within this dataset. Both types of EPS were represented by samples from diatom cultures as well as sediments. Based on this analysis, a conceptual model of EPS secretion was presented. This model proposed secretion of different groups of extracellular glycoconjugates, which could mainly be differentiated in (i) a light-dependent production of labile low molecular weight sugars, (ii) light-dependent production of a polymeric glucose-rich fraction closely attached to cells and, (iii) light-independent production of a more heterogeneous (colloidal) EPS that varied to some degree among different species.

In addition, it has become increasingly clear that an extracted EPS fraction does not necessarily contain one separate functional type of EPS but may consist of a mixture of different polymers. By using pyrolysis-mass spectrometry, Smith & Underwood (2000) showed differences in the composition of colloidal EPS produced during the logarithmic and stationary phase in cultures of 5 epipelic diatoms. The presence of two types of EPS in the colloidal EPS extracts of the diatom *Cylindrotheca closterium* were identified by precipitation of the EPS through a series of increasing alcohol concentrations (Underwood *et al.* 2004). In that study, nutrient replete cells produced a complex EPS while nutrient limited cells produced an additional EPS that had a less complex monosaccharide composition. Furthermore, examination of EPS-secretion by the diatom *Cylindrotheca fusiformis* under N- and P-limitation recovered at least three exopolymers with different size distributions of which two were different in monosaccharide composition (Magaletti *et al.* 2004). Wustman *et al.* (1997) observed that stalks produced by diatoms that caused fouling were composed of different parts that were apparently formed by distinctly different types of EPS. A detailed characterization of the water-insoluble, bicarbonate-extractable EPS-fraction of *A. longipes* showed the presence of polymers in three size ranges that were different in composition. Subsequent use of enzyme-linked immunosorbent assays (ELISA) showed the different locations of these different polymers, and indicated that their concerted action results in the formation of highly structured stalks.

By using a similar approach it was shown that two extracellular glycoproteins of a size >200 kDa secreted by the diatom *Craspedostauros australis* were involved in adhesion of the cell to a substratum as well as in cell motility while two smaller glycoproteins (87 and 112 kDa) formed a non-adhesive cell surface mucilage. The above mentioned examples not only show that benthic diatoms are able to secrete compositionally distinct types of EPS that may serve different functions, they also indicate that self assembly of extracellular components lead to the formation of complex structural biocomposites. It has been emphasized that the formation of these extracellular complexes rather than the secretion of a single substance are important for diatom functionality such as motility and adhesion to a substratum (Wetherbee *et al.* 1998).

Another important aspect of EPS-secretion concerns the uniformity of EPS-composition among different species of benthic diatoms. Variations in monosaccharide composition have been observed among EPS obtained from different benthic diatoms. Unfortunately, experimental conditions under which the diatoms were grown as well as the extraction protocols were not uniform. This might have influenced the outcome of the analyses of EPS and it is therefore not opportune to draw conclusions based solely on the comparison of monosaccharide composition. Only few studies have been published that used linkage analysis to identify substitution patterns in the EPS of diatoms (Chiovitti *et al.* 2003a; Chiovitti *et al.* 2003b; Wustman *et al.* 1997). In general, these investigations showed the presence of complex heterogeneous glycoconjugates. Comparison of glycoconjugates from different diatoms suggests that their structure is highly species specific.

Another approach is to combine destructive structural analysis of carbohydrate fractions from different diatom species and non-destructive visualization of glycoconjugates using lectins. Lectins are proteins that lack enzymatic or immunogenic activity and that possess specific carbohydrate and protein binding sites (Neu and Lawrence 1999). Lectins have mostly been characterized by its specificity for certain monosaccharides, although it has become increasingly evident that complex glycoconjugates are probably the more competitive binding sites. Monosaccharide compositions in four different carbohydrate fractions extracted from three different species of benthic diatoms are shown in Table 1. The soluble EPS fraction from all three species showed a heterogeneous distribution of monosaccharides with galactose, glucose, mannose/xylose and glucuronic acid being the most abundant sugars, each of which contributed >10% to the carbohydrate composition. In contrast, bound EPS and internal sugar fractions were composed almost exclusively of glucose which represented 75-96% of the carbohydrate. Also, the residual sugars were dominated by glucose or by xylose/mannose but this material showed a considerable variation among different species. Considering that the extracted fractions may represent mixtures of glycoconjugates it is difficult to judge if the material present in the extractants provide structurally unique exopolymers. In order to solve this problem, a set of 15 lectins was applied to the cultures of the 3 diatom species and the intensity of labeling and the localization of the labels were qualitatively evaluated (Table 2). The responses of the various lectins differed greatly among the extracellular matrices of the three species of diatoms. In addition, the localization of the

Table 1. Monosaccharide distributions of carbohydrate fractions extracted from early stationary phase cultures the diatoms *Cylindroteca closterium*, *Navicula mutica* and *Nitzschia brevissima*. Extraction procedure and monosaccharide analysis was conducted according to De Brouwer and Stal (2002) extended with direct methanolyses of the residual cell pellet to obtain the residual sugar fraction. Values represent averages and standard deviations of 3 replicate measurements.

Monosaccharide	Soluble EPS	Bound EPS	Internal sugars	Residual sugars
Cylindrotheca closterium				
Fucose	3.4 (0.2)	1.0 (0.2)	1.1 (0.7)	1.0 (0.2)
Rhamnose	14.3 (1.4)	7.0 (0.8)	2.8 (0.4)	5.1 (0.2)
Arabinose	0.5 (0.06)	0.2 (0.03)	n.d.	n.d.
Glucoseamine	1.3 (0.1)	0.1 (0.03)	0.1 (0.03)	0.5 (0.03)
Galactose	19.9 (2.7)	4.3 (0.2)	3.1 (0.6)	16.3 (0.5)
Glucose	13.8 (0.14)	77.7 (2.7)	86.7 (2.1)	64.4 (3.5)
Mannose/xylose	14.5 (0.3)	3.3 (0.4)	3.3 (0.3)	3.4 (2.4)
Galacturonic acid	5.3 (4.1)	1.5 (0.4)	1.2 (0.1)	3.4 (0.3)
Glucuronic acid	27.0 (0.5)	4.9 (1.3)	1.6 (0.2)	5.9 (0.7)
Navicula mutica				
Fucose	0.6 (0.04)	0.03 (0.03)	0.7 (0.2)	1.2 (0.2)
Rhamnose	9.5 (0.3)	2.5 (1.3)	n.d.	4.1 (0.03)
Arabinose	0.3 (0.01)	0.1 (0.01)	n.d.	n.d.
Glucoseamine	1.2 (0.07)	0.2 (0.1)	0.02 (0.03)	0.7 (0.06)
Galactose	11.0 (0.15)	1.4 (0.7)	1.6 (0.6)	17.7 (1.5)
Glucose	49.4 (1.2)	93.3 (3.0)	94.2 (2.2)	28.6 (6.5)
Mannose/xylose	11.7 (0.5)	0.7 (0.6)	3.5 (1.4)	43 (5.3)
Galacturonic acid	1.3 (0.4)	n.d.	n.d.	0.6 (1.0)
Glucuronic acid	15.0 (1.1)	1.8 (0.5)	0.05 (0.08)	4.2 (0.1)
Nitzschia cf. *brevissima*				
Fucose	7.3 (0.8)	3.4 (0.4)	n.d.	0.6 (0.2)
Rhamnose	7.9 (0.3)	3.6 (1.1)	n.d.	0.5 (0.1)
Arabinose	1.5 (0.1)	1.6 (0.2)	n.d.	n.d.
Glucoseamine	0.7 (0.09)	n.d.	n.d.	n.d.
Galactose	17.5 (0.6)	9.8 (1.8)	3.6 (1.4)	4.0 (0.4)
Glucose	21.0 (3.5)	75.2 (2.4)	96.0 (1.6)	91.3 (1.6)
Mannose/xylose	27.9 (1.4)	6.4 (2.0)	0.4 (0.3)	2.2 (1.1)
Galacturonic acid	4.0 (0.4)	n.d.	n.d	n.d.
Glucuronic acid	12.0 (1.8)	n.d.	n.d	1.5 (0.03)

Table 2. Response and localization of 15 different lectins applied to axenic cultures of the benthic diatoms *C. closterium*, *N. mutica* and *N.* cf. *brevissima*. Labelling response was assessed by qualitatively assessing the visualizations; X: no signal, –: low signal intensity, ±: intermediate signal intensity, +: good signal intensity, ++: excellent signal intensity. Localization coding; CS: cell surface, M: matrix staining, S: staining of bright extracellular spots, (p): partial staining.

Lectin	*C. closterium*		*N. mutica*		*N.* cf. *brevissima*	
Aleuria aurantia	+	CS	++	CS+M(p)	X	
Amaryllis	–	CS	±	CS(p)+M	–	CS(p)
Concanavaline A	±	CS	X		++	M
Helix aspersa	+	CS	X		+	S
Iberis amara	+	CS(p)	+	M(p)	++	CS+M+S
Lens culinaris	–	CS(p)	+	CS(p)+M(p)	++	M
Limulis polyphenus	X		X		-	CS(p)
Lycopersicon esculentum	X		-	CS	+	M+CS(p)
Maackia amurensis	X		X		-	S
Phaseolus coccineus	X		X		++	S+M
Sambuca nigra	X		X		X	
Solanum tuberosum	X		X		X	
Urtica dioica	X		+	CS	-	CS
Vicia sativa	–	CS	++	CS	±	CS(p)+S
Wheat germ agglutinin	X		+	CS+M(p)	X	

labels was distinctively different (Table 2, Figure 1). These results strongly suggest that the extracellular material secreted by these benthic diatoms contained highly specific glyconjugates. This agrees with earlier work that showed that different types of EPS may have a specific localization within the extracellular matrix of diatoms (Lind *et al.* 1997; Wustman *et al.* 1998).

Function and mechanism of EPS-secretion

Diatoms secrete substantial amounts of photosynthetically fixed carbon as extracellular carbohydrates. Estimates reported for epipelic diatoms range between 30-73% of the fixed CO_2 being secreted as EPS (Goto *et al.* 1999; Middelburg *et al.* 2000; Smith & Underwood 2000; de Brouwer *et al* 2000; Wolfstein *et al.* 2003). In epipelic diatoms EPS is secreted from a long narrow slit (raphe) in the silica frustule. Edgar & Picket-Heaps (1984) suggested that the role of EPS in gliding movement was to attach the diatom to a substratum. They conceived that the EPS strands were displaced parallel to the raphe as a result of contractions of microfilaments of the cytoskeleton, which would provide the force for gliding. Subsequently, Webster *et al.* (1985) identified this

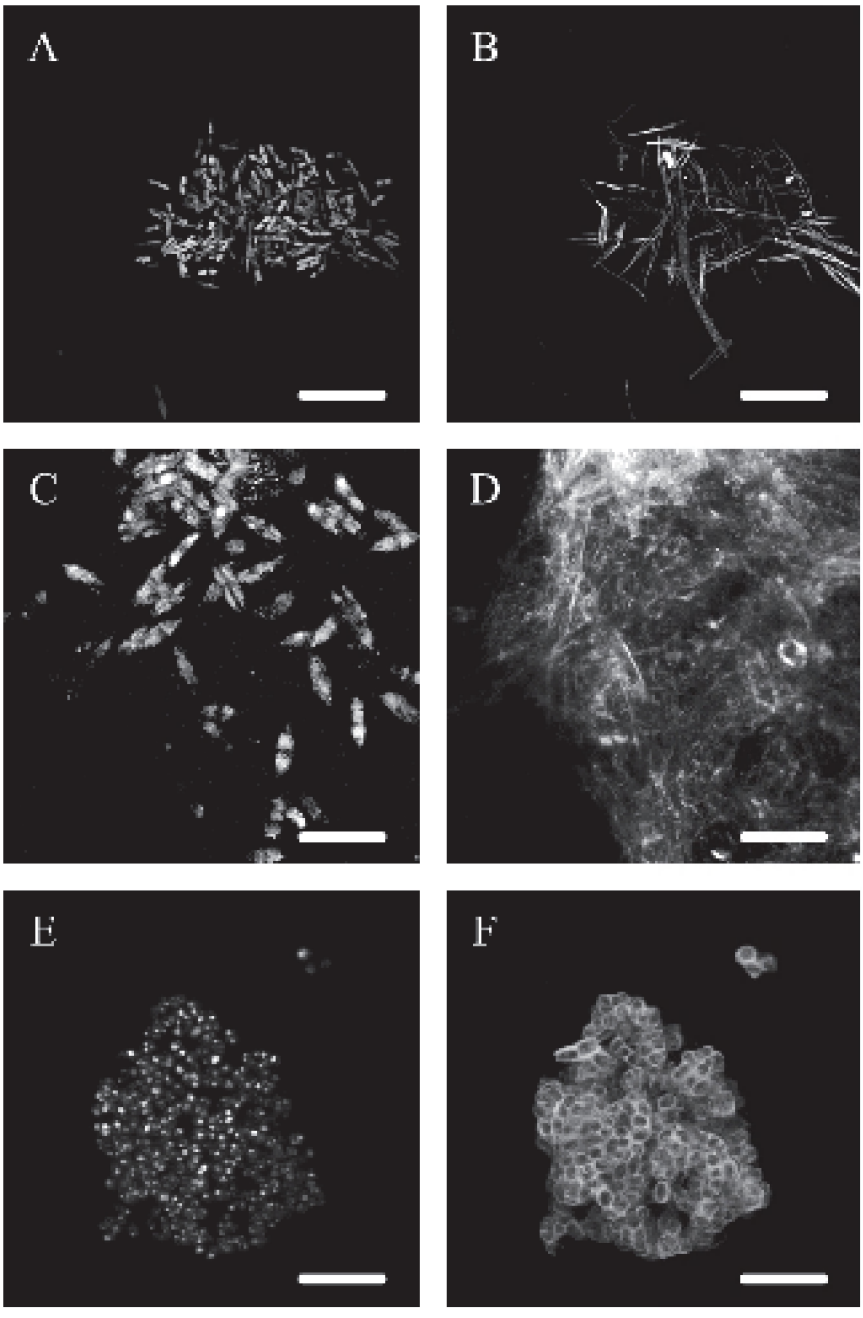

Figure 1. Maximum intensity images of chlorophyll *a* (A, C, E) and lectin signal (B, D, F,) of aggregates from axenic cultures of the diatoms *Cylindrotheca closterium* (A, B) stained with *Iberis amara*, *Nitzschia* cf. *brevissima* (C, D) stained with concanavaline A and *Navicula mutica* stained with the lectin *Iberis Amara*. Scale bars: A-D: 40 μm; E-F: 20 μm.

EPS as actin- and tubuline-based microfilaments that were involved in gliding motility of the diatom *Amphora coffeaeformis*. This role of EPS was experimentally confirmed by Lind *et al.* (1997) who used antibodies to inhibit substratum adhesion and gliding of the diatom *Stauroneis decipiens*. Some researchers have observed that diatoms in intertidal mudflats migrate in response to light and to the tide (Paterson 1986; Pinkney & Zingmark 1991; Serôdio *et al.* 1997; Smith & Underwood 1998; Underwood & Smith 1998a), although others did not observe such migration so that it does not seem to be a general mechanism. Since diatoms also migrate in dark, EPS secreted for motility must occur independent of light. That this is indeed the case has been demonstrated in cultures (Smith & Underwood, 2000; de Brouwer & Stal 2003) as well as under natural conditions in intertidal mudflats (Smith & Underwood 1998).

Hitherto, the amount of EPS required for gliding has not been quantified but Edgar & Pickett-Heaps (1984) conceived that motility probably requires only small quantities of EPS, and hence, would represent a low metabolic expense. Since benthic diatoms secrete large amounts of EPS it is attractive to suppose that the production of this material serves other purposes. Because it was found that EPS production was light dependent and coupled to photosynthesis (de Winder *et al.* 1999; Staats *et al.* 2000a) it was conceived that it was the result of unbalanced growth caused by the depletion of an essential nutrient. This view is supported by the results from culture experiments (Lewin 1955; Myklestad & Haug 1972; Bhosle *et al.* 1995; Staats *et al.* 2000b) that demonstrated that nutrient depletion (particularly nitrogen and phosphorous) enhanced exopolymer production. Mass balance calculations agreed also with the occurrence of unbalanced growth due to nutrient shortage (Ruddy *et al.* 1998a). By coupling nitrogen limitation to carbohydrate production, the model of Ruddy *et al.* (1998b) accurately described short-term microphytobenthos and exopolymer dynamics in intertidal mudflat sediments.

Currently, hardly any information is available explaining the mechanisms by which EPS is secreted and describing the possible pathways of its production. By using radioactive labeled $^{14}CO_2$, it was shown that the transfer of photosynthetically fixed carbon into the extracellular pools occurred within 30 min. This was shown both for a culture of *C. closterium* as well as in a field sample of microphytobenthos (Wolfstein *et al.* 2002). This suggests a close coupling between photosynthesis and EPS secretion as was already emphasized by de Winder *et al.* (1999) and Staats *et al.* (2000a). However, this was not confirmed by Underwood *et al.* (2004), who reported for the same species that the flow of photosynthetically fixed carbon to EPS occurred with a delay of 3-4 h, suggesting an uncoupling of photosynthesis and EPS-production. By using inhibitors that block glucan synthesis, glucan catabolism and protein synthesis, Underwood *et al.* (2004) suggested at least two pathways for EPS production. One pathway was dependent on the synthesis and subsequent catabolism of intracellular glucan, while the other pathway was independent of glucan catabolism. This is strong evidence that diatoms may excrete different types of EPS as the product of different metabolic pathways and that therefore follow different dynamics and differ in composition (see previous section).

Other parameters may also affect the production of EPS. Cooksey (1981) for instance demonstrated attachment of diatoms to a surface was inhibited in the absence of Ca^{2+}. This was accompanied by a large decrease in EPS-secretion (K.E. Cooksey, personal communication). Moreover, it was observed that adjustment of the salinity of the medium had an effect on growth and EPS release in diatom cultures (Tokuda 1969; Allan *et al.* 1972) and also changed the composition of the EPS (Allan *et al.* 1972). Furthermore, effects of light levels and temperature may alter the production of extracellular material. Staats *et al.* (2000a) observed that short-term production of EPS occurred above a photon irradiance of 15 μmol $m^{-2}s^{-1}$ and they suggested that a certain minimum amount of light was required to allow accumulation of exopolysaccharides. However, radioactive ^{14}C-labelling experiments over a broad range of photon irradiances (7-1200 μmol $m^{-2}s^{-1}$) resulted in only labeled EPS, and Wolfstein *et al.* (2002) concluded that this light threshold for EPS-production must have been below 7 μmol $m^{-2}s^{-1}$. Temperature is another factor that affects EPS production. Wolfstein and Stal (2002) found that the rates of carbon fixation and EPS excretion were maximal at 25 °C. This value agrees with the optimum temperature for photosynthesis in microphytobenthos (Blanchard *et al.* 1996). Light and temperature affected the patterns and quantities of the different EPS-fractions (i.e. colloidal and attached EPS) in different ways, which once again suggest that these fractions are produced and secreted by different pathways (De Brouwer and Stal 2002).

The roles of EPS in intertidal coastal areas

Sediment stabilization

EPS produced by benthic assemblages in intertidal mudflats plays an important role in stabilization of surface sediments. Several studies have shown the relation between the presence of diatom biofilms and a decrease in sediment erosion (De Brouwer *et al.* 2000; Paterson 1989; Sutherland *et al.* 1998; Underwood and Paterson 1993). It is, however, not easy to experimentally verify the specific role of EPS in the mediation of biogenic sediment stabilization, and experiments often lead to contradictory results. For example, Underwood and Paterson (1993) found that colloidal carbohydrate was the best biochemical predictor of sediment stability, but other studies indicated that no such relation existed (De Brouwer *et al.* 2000; Defew *et al.* 2002; Paterson *et al.* 2000). (Yallop *et al.* 2000) found that sediment stability correlated with several variables including chlorophyll *a*, extracellular carbohydrate and EPS fractions, water content and bacterial biomass. By using multiple regression analysis these authors calculated that sediment stability was best predicted by using a combination of chlorophyll *a*, colloidal EPS and water content. This indicates that multiple processes and their interactions are involved in the process of biogenic stabilization in intertidal sediments. Biogenic stabilization is the result of the secretion of EPS that leads to the formation of a cohesive organic matrix in which diatoms and sediment particles are embedded (Taylor and Paterson 1998). Besides the physical effect of EPS as a 'glue' by which sediment particles stick together, a decrease in bottom roughness resulting

from the formation of the biofilm probably has an additional effect on sediment stabilization (Paterson and Black 1999).

An approach that directly assesses the role of EPS in sediment stabilization is to add it to sediment and subsequently measure changes in sediment behavior. Several studies have demonstrated that bacterial EPS modified sediment characteristics (Dade *et al.* 1990; Tolhurst *et al.* 2002). De Brouwer *et al.* (2002a) and Perkins *et al.* (2004) used EPS that was previously extracted from intertidal sediments and subsequently added to sediments in known amounts. De Brouwer *et al.* (2005) used EPS from pure cultures of various benthic diatoms and added it to muddy sediments. Surprisingly, EPS derived from benthic diatoms or from natural diatom biofilms did not affect sediment characteristics in the way it was shown for bacterial EPS. Diatom-derived EPS partly adsorbed to sediment particles but did not change the sediment properties (De Brouwer *et al.* 2002a). Calcium-ions enhanced the adsorption of EPS, which suggests that cation-bridging is an important mechanism to bind EPS to sediment particles. Furthermore, it was shown that uronic acids play a role in sediment-EPS interactions (Dade *et al.* 1990; De Brouwer *et al.* 2005), which further emphasizes the importance of cation bridging in adsorption of EPS to the sediment. Perkins *et al.* (2004) found that the addition of EPS affected sediment properties when it was subjected to desiccation, its overall effect being an increase in the threshold of sediment erosion, probably because of increased electrostatic binding between sediment particles (Chenu 1993). The addition of EPS resulted in an increased retention of water while erosion thresholds were comparable to controls. The interpretation of these results was difficult because it was observed that the presence of salts alone (naturally present in the EPS extracts) also led to increased sediment stability.

Although it was not possible to show convincingly that EPS alone is able to stabilize mud, this does not necessarily exclude its role in sediment morphogenesis. It should be taken into account that EPS in the biofilms forms structured entities (Decho 1994) and it is likely that this structure is lost upon extraction (De Brouwer *et al.* 2002a; Underwood and Paterson 2003). Visualization of diatom EPS using carbohydrate-specific lectins (Figure 1) showed that exopolymers are present as ordered structures. For the diatom *C. closterium* only cell surface glycoconjugates were visible, while for the other two benthic diatom species the set of lectins also visualized matrix-structures. The morphological characteristics of the matrix glycoconjugates varied between species. *N. mutica* formed a honeycomb type of structure where the algae where associated mainly with EPS that was located closely to the organisms. In contrast, *N.* cf. *brevissima* formed an extensive matrix existing of channels as well as amorphous structures. Because the composition and structure of EPS varies among diatoms, it is likely that biogenic sediment stabilization is also species dependent. Culture experiments have confirmed this (De Brouwer *et al.* 2005; Holland *et al.* 1974). Furthermore, the relations between extracellular carbohydrate fractions and critical shear stress for axenic cultures of the diatoms *Cylindrotheca closterium* and *Nitzschia* cf. *brevissima* showed that, although these species secreted similar amounts of EPS, the effect on the rheological properties (i.e. critical shear stress) of the sediments were notably different. Hence, it was concluded that the addition of isolated EPS from these diatoms did not affect sediment stability. It was conceived that in

addition to the quantity and chemical composition of the EPS, the assembly of exopolymers in ordered three dimensional matrix structures is essential to increase the erosion threshold of muddy sediments.

Currently, it is unknown which fraction of EPS is actually responsible for sediment stabilization. It has been suggested that EPS related to motility is important for the stabilization of intertidal sediment (Paterson 1989). On the one hand, extracellular polymers that putatively serve for motility and adhesion to a substratum are highly cohesive (Higgins *et al.* 2002), and this suggests that it could glue sediment particles. On the other hand, it is known that motility trails of cultured diatoms usually detach shortly after secretion and are not likely to form such extended structures as those depicted in Figure 1. This view was confirmed by Wigglesworth-Cooksey and Cooksey (2004) who noted that the footpath and motility extracellular glycoconjugates produced by *Amphora coffeaeformis* and a *Navicula* sp. appeared to have an effect only over short distance in order to establish a physical contact between the diatom and a substratum (Wetherbee *et al.* 1998). However, in addition polymers with a different composition were secreted forming an extensive matrix. This EPS was more likely to mediate sediment stabilization because it exerted its effect over much greater distances and was potentially able to embed sediments into the EPS-matrix. By using a model sediment system, Wigglesworth-Cooksey and Cooksey (2004) also observed that sediment stability (measured as hydraulic conductivity) induced by *A. coffeaeformis* was closely correlated with the accumulation extracellular matrix material that was not soluble in 0.5 M $NaHCO_3$ at 90 °C (Wigglesworth-Cooksey *et al.* 2001). This polymer was mainly produced under PO_4^3-limiting conditions, suggesting that nutrient limitation could play a role in secretion of matrix EPS and thus sediment stabilization. Similarly, the erosion rate of a sediment inoculated with a culture of the diatom *Nitzschia curvilineata* was highly correlated with the bulk carbohydrate to chlorophyll *a* ratio, which was considered as an indicator of the physiological state of the diatoms (Sutherland *et al.* 1998). Also in this study stationary phase conditions appeared to trigger production of bulk EPS as well as the decrease in erosion rate.

EPS as a food source

Exopolymers are a potential food source for other organisms inhabiting intertidal environments. In general, utilization of organic carbon by bacteria occurs rapidly. When extracellular fractions originating from a community of benthic diatoms were added to sediment slurries, it was found that 50 % was utilized within 24 h (Goto *et al.* 2001). For extracellular polymers isolated from the pelagic diatom *Thalassiosira* a decrease of 50 % in EPS concentration was obtained after 11-25 days of incubation (Aluwihare and Repeta 1999; Giroldo *et al.* 2003). The difference in this utilization rate between the pelagic and benthic systems is perhaps the results of a much higher density of bacteria in the sediment. The degradability of extracellular organic matter is dependent on its composition and structure. Nevertheless, Goto *et al* (2001) found little variation in the decomposition of various extracellular carbohydrate fractions that were isolated from different diatom species as well as a natural microphytobenthos assemblage. This indicates that compositional variations in the extracellular

material were not limiting the initial degradation of the extracellular carbon pool, suggesting that a wide variety of carbohydrate hydrolyzing enzymes may be present in sediments to enable a rapid degradation of bioavailable glycoconjugates.

Also *in situ* studies in intertidal mudflats indicate that transfer of algal derived extracellular material occurs rapidly. Middelburg *et al.* (2000) showed that the transfer of photosynthetically fixed carbon into bacterial phospholipid fatty acids occurred within 4 h. Although these authors did not specifically investigate the role of EPS they concluded that transfer of organic carbon to bacteria occurred via extracellular material, which was estimated to represent 40% of photosynthetically fixed carbon. This confirms the observation that a short-term coupling exists between bacterial production and extracellular compounds released by diatoms in phototrophic biofilms (Van Duyl *et al.* 1999). Water-extractable carbohydrates appeared to play an important role in bacterial dynamics. Its rapid utilization by diatoms suggests that this water-extractable carbohydrate represents a highly labile pool of carbon. Indeed it was found that the EPS produced in the surface layer of the sediment during tidal emersion consisted of polymers that consisted predominantly of glucose (De Brouwer and Stal 2001; Taylor *et al.* 1999). Glucose is preferentially degraded by bacteria (Giroldo *et al.* 2003; King 1986) indicating that this photosynthetically produced EPS fraction is important for transfer of carbon within the microbial foodweb.

Utilization of EPS is not necessarily restricted to bacteria. Heterotrophy has been observed among diatoms and some observations indicate that diatoms decompose their own EPS, utilizing it as an energy storage when deprived of light (Smith and Underwood 2000; Staats *et al.* 2000). Furthermore, meio- and macrofauna may also utilize EPS, however not much information on this subject is available. Current knowledge (Decho and Lopez 1993; Hoskins *et al.* 2003) suggest that certain animals were able to efficiently utilize algal as well as bacterial EPS. However, it should be emphasized that EPS is a poor food source with respect to its nutritional value. It is rather a valuable energy source in combination with other, more nutritional, compounds. Further research is necessary to identify the different consumers that utilize this labile carbon source in intertidal environments.

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