Journal of Sea Research 92 (2014) 26–35

Contents lists available at ScienceDirect

Journal of Sea Research

journal homepage: www.elsevier.com/locate/seares

Seasonal dynamics of extracellular polymeric substances (EPS) in surface sediments of a diatom-dominated intertidal mudflat (Marennes–Oléron, France)☆

Guillaume Pierre [a,b,*], Jean-Michel Zhao [b], Francis Orvain [c], Christine Dupuy [b], Géraldine L. Klein [b], Marianne Graber [b], Thierry Maugard [b]

[a] Clermont Université, Université Blaise Pascal, Institut Pascal, UMR 6602 CNRS. Polytech Clermont-Ferrand, 24 avenue des Landais, BP206, F-63174 Aubière cedex, France
[b] UMR 7266 CNRS, ULR LIENSs, Equipe Approches Moléculaires Environnement Santé, Université de La Rochelle, UFR Sciences, Bâtiment Marie Curie, avenue Michel Crépeau, 17042 La Rochelle, France
[c] Université de Caen Basse-Normandie, CNRS FRE 3484 BioMEA, Esplanade de la Paix, 14032 Caen, France

ARTICLE INFO

Article history:
Received 7 December 2012
Received in revised form 25 March 2013
Accepted 23 July 2013
Available online 5 August 2013

Keywords:
Extracellular Polymeric Substances (EPS)
Carbohydrates
Diatoms
Seasonal Impacts
Mudflat

ABSTRACT

Numerous field-based investigations have highlighted that the production of extracellular polymeric substances (EPS) is physico-chemically and ecologically important for intertidal mudflats. EPS are largely secreted by marine benthic diatoms and their quantity and quality are environmental-dependant. This paper focused on the dynamic pathways, concentration rates and monosaccharides composition of colloidal, bound and residual carbohydrates extracted by using a cationic exchange resin from a diatom-dominated intertidal mudflat (Marennes–Oléron, France) during two different sampling periods: winter (February 2008) and summer (July 2008). A wide range of biotic and abiotic parameters were also studied to better understand the effect of environmental parameters, e.g., chlorophyll *a*, salinity, pore water amount, emersion time, luminosity, C:N ratio and tidal coefficient. Multiple colorimetric assays coupled to gas chromatographic analyses were carried out to perform the biochemical characterizations. Firstly, the quantity of carbohydrates produced during winter (5.28 μg·μg chl a^{-1}) was more important than during summer (2.04 μg·μg chl a^{-1}). Yet, more proteins were found during summer for the colloidal and bound fractions (0.73 and 1.04 μg·μg chl a^{-1}). Further investigations showed that the dynamic pathways were equivalent between winter and summer: bound carbohydrates (BC) quantities increased during the sediment emersion periods on the contrary to colloidal carbohydrates (CC) which tended to drop throughout the emersion time. The quality in monosaccharides was fraction-dependant, whatever the season. CC were always glucose-rich confirming their role of carbon source. BC were mainly composed of rhamnose whose the ratio increased during the emersion period, thus conferring adhesive properties to the extracellular matrix bounding diatoms cells. Residual carbohydrates (RC) were composed of various monosaccharides and a major increase of glucose content was found at the end of emersion, corresponding to intracellular C-storage in prevention to immersion times. Summer-RC were composed of fucose, a monosaccharide specific to these fractions and which was non-present during the winter campaign. Environmental parameters, such as salinity, pore water amount, and tidal coefficient could have a significant impact on the concentration rates and pathways of carbohydrates.

1. Introduction

Intertidal mudflats are extremely productive areas and may provide up to 50% of the primary and secondary production of estuaries (Underwood and Kromkamp, 1999), especially due to the formation of diatom-dominated biofilms at their surface (Falciatore and Bowler, 2002). Subject to periodic tidal exposure, the physical and chemical properties of these biofilms change (Christie et al., 2000; de Brouwer and Stal, 2001). Thus, some authors noted that variations in sediment dynamics and compositions could result from seasonal fluctuations (Frostick and McCave, 1979), and particularly Extracellular Polymeric Substances (EPS). Benthic biofilms are composed of water, microalgae, prokaryotes (bacteria, archaea), other eukaryotic microbes, virus and inorganic particles entangled in an EPS matrix Yallop et al. (2000). This EPS matrix is rich in a wide variety of polysaccharides, proteins, glycoproteins, lipids and nucleic acids (Wingender et al., 1999). Besides, marine diatoms are known to produce various kinds of EPS depending on the environmental conditions and the tidal periods (Underwood et al., 2004). On one hand, colloidal EPS are excreted in the micro-environment of the benthic biofilms (Underwood et al.,

☆ Given her role as Guest Editor/Editor-in-Chief Christine Dupuy had no involvement in the peer-review of this article and have no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Hélène Agogué.

* Corresponding author at: Clermont Université, Université Blaise Pascal, Institut Pascal, UMR 6602 CNRS, Polytech Clermont-Ferrand, 24 avenue des Landais, BP206, F-63174 Aubière cedex, France. Tel.: +33 4 73 40 74 22; fax: +33 4 73 40 78 29.
E-mail address: guillaume.pierre@univ-bpclermont.fr (G. Pierre).

1385-1101/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.seares.2013.07.018

1995) and are used by different micro-organisms, e.g., the glucose-rich exudates consumed by heterotrophic bacteria (Hofmann et al., 2009; van Duyl et al., 1999). On the other hand, bound EPS are secreted as mucilaginous slime coating the cells (Underwood and Paterson, 2003). These EPS play key roles in microbial and physico-chemical defense (Decho, 1990), in the motility of epipelic diatoms (Stal and Défarge, 2005), in cells/cells or cells/substratum adhesion (Wimpenny et al., 2000) and sediment biostabilization (Spears et al., 2008). Several studies have reported large variability of EPS composition, abundance and properties, thus highlighting the scientific challenge to extract and analyze consistently EPS by biochemical methods (Azerado et al., 2003; de Brouwer and Stal, 2004). In this way, Underwood and Paterson (2003) have pointed the importance of the biochemical methodology to accurately reflect the biological utility or relevance of EPS in benthic biofilms. Recently, an alternative method for in situ EPS extraction and determination (Takahashi et al., 2009), applied during two field studies (Pierre et al., 2010, 2012), has shown the possibility to separate three major classes of EPS through the use of a cationic exchange resin (Fig. 1). Moreover, the use of diluted polar solvents in these works allowed the size separation of EPS: short-chain to long-chain oligosaccharides, i.e. low molecular weight (LMW) compounds and high molecular weight (HMW) compounds (Bellinger et al., 2005; Decho, 2000). Such characterization of EPS is a necessary step to determine and understand their roles in benthic biofilms, especially if we focus on the influence of biotic and abiotic parameters.

The goal of this study was to compile numerous data obtained on the same field to investigate the temporal distribution and compositional changes of EPS, in relation to seasonal dynamics. The EPS samples were collected from a diatom-dominated biofilm (Marennes–Oléron Bay, France) during different ecological periods in the chemistry and biology of tidal flats, i.e. in February 2008 (winter) and July 2008 (summer), when the micro-phytobenthic biofilm development and its EPS composition are supposed to be drastically dissimilar.

2. Material and methods

2.1. Field sampling

The sediment samples were collected from Marennes–Oléron Bay (Atlantic Coast of France), during only one week in February 2008 (winter) due to strong field constraints and two weeks in July 2008 (summer) at low tide (Fig. 2). The field sampling was organized as a chessboard where square sampling units (2 m-side) separated by alleys (2 m in width) were defined. Every day, three squares were randomly considered for spatial heterogeneity. Sediment samples from each square were collected by using core diameter of 20 cm. Three cores of each selected square were sampled every hour during the tidal periods. For each core, the top 1 cm was collected three times and pooled. After each sampling, sediment pools were brought back from the field for an immediate EPS extraction on fresh sediments. Biochemical analyses were then performed in triplicate on the colloidal, bound and residual fractions (864 fractions).

2.2. Biotic and abiotic measurements

The chlorophyll *a* concentration in the sediment was measured using fluorometry method (Lorenzen, 1966). Light was measured using a Li-Cor sensor which was recorded every minute during sampling days. Salinity, pore water amount, and C:N ratio were also followed. Enumeration of bacteria was performed by microscopy, after 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) labeling (×1000, Axioskop, Zeiss) using the method of Porter and Feig (1980). All missing data were due to experiment constraints.

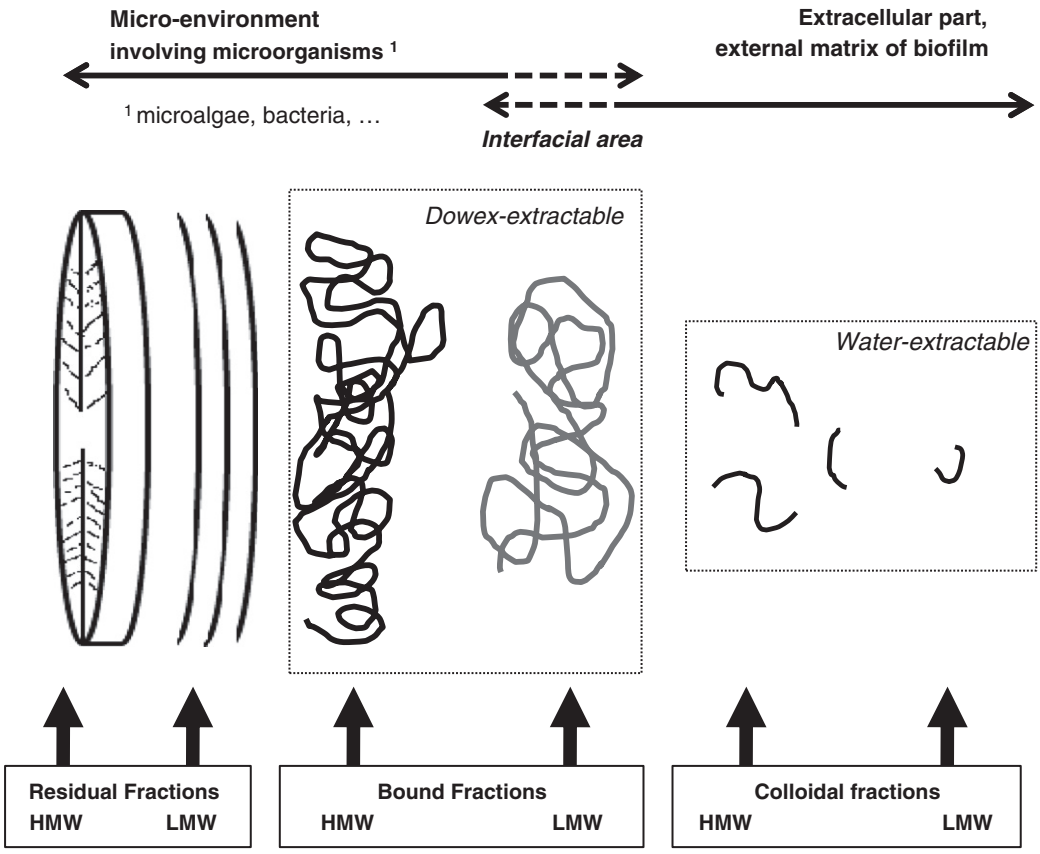

Fig. 1. Location hypothesis of the different EPS fractions collected by the Dowex method, adapted from the model of Underwood and Paterson (2003).

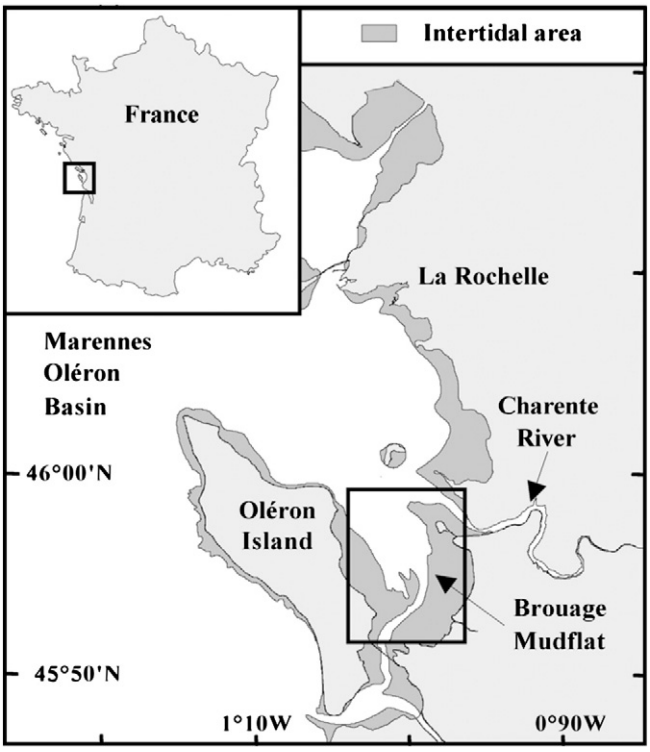

Fig. 2. Location of the study area (Brouage mudflat, France) where samples of surficial intertidal sediments were collected.

2.3. Material

Solvents, cationic resin (Dowex Marathon C), assay kits, protein (Bovine Serum Albumin, BSA) and carbohydrate standards (dextran, dextran sulfate, glucose, galactose, rhamnose, fucose, fructose, xylose, arabinose, ribose, mannose, *myo*-inositol, glucuronic and galacturonic acids) were obtained from Sigma-Aldrich. The DB-1701 J and W Scientific column (30 m, 0.32 mm, and 1 μm) for gas chromatography–mass spectrometry analysis (GC/MS) was obtained from Agilent.

2.4. EPS extraction and fractionation

The EPS extraction method (Takahashi et al., 2009) was done immediately after sampling and sediment mixing on the field. 20 mL of fresh mudflat was continuously mixed with 20 mL of Artificial Sea Water (ASW 30 practical salinity units) for 1 h in darkness at 4 °C and then centrifuged at 3500 ×g and 4 °C for 10 min. The supernatant containing colloidal EPS was collected and stored at 4 °C. 20 mL of ASW and 1 g of activated Dowex Marathon C, previously prepared in phosphate buffer saline for 1 h in the dark, was added to the sediment pellet. The samples were mixed gently at 4 °C for 1 h in the dark and then centrifuged at 3500 ×g and 4 °C for 10 min. A supernatant containing the bound EPS and a cap containing intracellular and residual polymers were obtained. The cap was then frozen. The residual polymers were extracted from the frozen sediment samples by sonication at 100 W for 3 min on ice after resuspension in 20 mL in ASW. For each fraction (colloidal, bound and residual polymers), absolute ethanol at −20 °C was added to the sample to obtain a final ethanol concentration of 75% (v/v). The solution was gently mixed and stored overnight at −20 °C. The solution was then centrifuged at 3500 g and 4 °C for 15 min to obtain a supernatant (LMW fraction) and a precipitate pellet (HMW fraction). Finally, the fractions were dried under air flow and stored at −20 °C.

2.5. Carbohydrate, uronic acid and protein analysis

Total sugar content was determined by the phenol–sulfuric acid assay, using glucose as a standard (Dubois et al., 1956). Total sugar amounts for the fractions were measured and normalized to chlorophyll *a* (chl *a*), thus allowing the overestimation of diatom EPS, comparing to other EPS sources (Underwood and Paterson, 2003). Uronic acid content was determined using the meta-hydroxydiphenyl method (MHDP), with galacturonic and glucuronic as standards (Blumenkrantz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991). Protein content was determined by the bicinchoninic acid (BCA) method, using bovine serum albumin (BSA) as a standard (Smith et al., 1987).

2.6. Gas chromatography coupled to mass spectrometry (GC/MS) to characterize the carbohydrate of EPS fractions

EPS fractions were solubilized in 5 mL of ultrapure water, dialyzed (6–8 kDa) and freeze-dried. EPS were then dissolved in 2 M HCl at 50 mg/mL and heated at 90 °C for 4 h. The preparation was then freeze-dried and stored at −20 °C. Analyses were carried out by GC/MS using a Varian CP-3800 GC/Varian Saturn 2000. 400 μL of pyridine and 400 μL of BSTFA: TMCS (99: 1) was added to 2 mg of purified monosaccharides. The solution was mixed for 2 h at room temperature, then injected into a DB-1701 J and W Scientific column (30 m, 0.32 mm, and 1 μm) at a flow of 1 mL/min. The helium pressure was 8.8 psi. The temperature of the injector was set at 250 °C. The rise in temperature in the oven was programmed for a first step at 150 °C for 0 min, then an increment of 10 °C/min up to 200 °C with a final step at 200 °C for 35 min. The ionization was performed by Electronic Impact (EI, 70 eV), the trap temperature was set at 150 °C and the target ion was fixed at 40–650 m/z.

2.7. Statistical analysis

All statistical analyses were run using the statistical software XLStat (Addinsoft). One-way ANOVA was used to analyze changes in carbohydrate and uronic acid amounts among abiotic parameters for each day (sampling location and emersion time). Data transformations (root) were performed to check application conditions (normality) each time it was required. Post hoc procedures (Tukey test) were performed to analyze pairwise differences. *t* and *Z*-tests were conducted to determine significant differences between values of variables at the beginning and at the end of the emerged period. Pearson correlation tests were done with the complete data set to investigate the relationships between the different EPS fractions with biotic (bacterial abundance, Chl *a*) and abiotic (luminosity, salinity) parameters. Principal component analyses were used to highlight and group fractions with closed monosaccharides distributions.

2.8. Abbreviations and nomenclature

In accordance with the literature (Underwood et al., 2010), terms and abbreviations are defined in Table 1.

3. Results and discussion

3.1. Relationships between EPS and environmental parameters: a seasonal heterogeneity?

The total carbohydrate quantities and the contribution of neutral carbohydrates, uronic acid and proteins were determined for the three main EPS fractions extracted in winter and summer (Table 2). At this step, it is noteworthy that the sugar amounts were normalized to Chl *a* in order to overestimate diatom EPS levels, compared to other EPS sources (Haubois et al., 2005). This normalization is classically used in the literature especially when the mudflat is mainly composed of micro-phytobenthos and artificially allows considering that EPS are

Table 1
Abbreviations and nomenclature.

Terms and abbreviations	Definitions	Method used
Carbohydrates	Total sugars	Dubois assays, GC/MS analyses
Uronic acids	Uronic acids	BCA assays, GC/MS analyses
CC	Colloidal carbohydrates	Water-extractable
BC	Bound carbohydrates	Dowex Marathon C extraction
RC	Residual carbohydrates	Sonication
LMW	Low molecular weight	Ethanol −20 °C (75%, v/v) centrifugation
HMW	High molecular weight	
W-	Winter	Sampling period 1
S-	Summer	Sampling period 2

mainly produced by diatoms (e.g., Oléron Bay mudflats). In average and regardless the emersion time, more colloidal carbohydrates were extracted from the sediment in winter (5.28 μg·μg chl a^{-1}) than summer (2.04 μg·μg chl a^{-1}). The same observations were done for the bound and residual carbohydrates since 2.5 times more carbohydrates were found in the winter samples (Table 2). Concerning the relative contribution of neutral and acidic sugars, the results showed that neutral sugars were the main component of the fractions both in summer and winter although the ratios varied during these two periods. For the colloidal and bound fractions, the neutral sugar ratios dropped from 78% to 61% and 87% to 63% respectively (relative % w/w) due to the presence of proteins in the fractions extracted in summer.

However, if we focus only on the carbohydrate part, no significant change ($p < 0.01$) was observed for the neutral sugar ratios. Previous papers noted that variations in sediment dynamics resulted from seasonal changes in biological influences (Frostick and McCave, 1979), EPS quality and quantity being excellent indicators to measure the impact of these modifications. In this way, correlations were found between the level of EPS and the diatom biomass in the sediment, i.e. EPS quantities reduced when the diatom biomass decreased (Staats et al., 2001). Ambient light climate play an essential role on the photosynthetic power of epipelic diatoms, which move up to the sediment (Serôdio et al., 1997). Micro-phytobenthic biofilms can have high rates of photosynthesis and a large part of the photo-assimilated carbon is excreted into the environment as exopolymers (Underwood and Paterson, 2003). During our sampling periods, the luminosity was around 1600 ± 400 μmol photons·m^{-2}·s^{-1} in summer and 440 ± 250 μmol photons·m^{-2}·s^{-1} in winter. The level of chl *a* measured on the sediment was significantly lower ($p < 0.01$) in summer (8.1 ± 1.2 μg·g dry $sediment^{-1}$) than in winter (20.2 ± 0.9 μg·g dry $sediment^{-1}$). This difference could be attributed to the intense level of grazing by *Peringia ulvae* whose the density was higher in summer (17.2 ind·m^{-2} ± 7.08) than in winter (5.77 ind·m^{-2} ± 2.99) but further experiments could be performed to confirm this hypothesis. Moreover, the classical normalization with chl *a* enhanced the gap between the values in summer and winter.

Table 2
Normalized composition (μg·μg chl a^{-1}) of the different fractions extracted from the Marennes–Oléron mudflat during winter and summer sampling campaigns.

Fraction	Total carbohydrate content	Uronic acid content	Protein content
Winter			
Colloidal fraction	5.28 ± 1.1	1.15 ± 0.54	0
Bound fraction	7.03 ± 2.2	0.89 ± 0.18	0
Residual fraction	56.5 ± 19	17.1 ± 3.89	16.2 ± 4.6
Summer			
Colloidal fraction	2.04 ± 0.9	0.36 ± 0.13	0.73 ± 0.5
Bound fraction	2.61 ± 1.3	0.27 ± 0.07	1.04 ± 1.3
Residual fraction	23.1 ± 8.6	7.23 ± 1.62	6.85 ± 2.8

±: deviations calculated from the heterogeneity of the sampling squares.

Besides, intertidal mudflats are living areas whose the primary trophic production is non-negligible. As an example, intertidal mudflats are productive areas which provide up to 40–50% of the primary production of estuaries (Underwood and Kromkamp, 1999; Underwood and Paterson, 2003). So, biota living in the intertidal zone are adapted to use EPS as food or as protection against environmental dynamics which dominate tidal flats (Widdows and Brinsley, 2002). During summer periods, consumers of EPS (carbon source) are much more active and directly responsible in a drop of sediment stability (Andersen, 2001). This greater summer consumption could also explain the differences between the amounts of carbohydrates measured in summer and winter.

Thirdly, the amounts of proteins detected during summer were interesting because specific of this period (Table 2). Yet, proteins were also found in colloidal and bound fractions collected in summer. During summer, pore water amount in the sediment was significantly lower than in winter ($p < 0.05$) and positive correlations were found with S-LMW CC and S-LMW BC (Table 3, $p < 0.05$). Some authors have suggested that EPS (carbohydrates and proteins) could protect micro-phytobenthos from high desiccation levels and salinity (Spears et al., 2008). Thus, we concluded that the presence of proteins combined to specific carbohydrates during summer could be a response to desiccation stress, explaining the positive correlations found (Table 3). These fractions could be correlated to osmoregulatory and hydratation properties to fight against extensive salinity. Besides, proteins can also be used as additional C-storage which can explain their presence in residual fractions, composed of intracellular (chrysolaminaran) and refractory EPS.

A number of authors have pointed at the rapid changes (hour), in term of colloidal carbohydrate amounts, which occur at the surface sediments during periodic tidal exposures (Christie et al., 2000; Hofmann et al., 2009; Pierre et al., 2012; Taylor and Paterson, 1998). The fate of colloidal and bound carbohydrate amounts between the beginning and the end of the emersion periods during winter and summer was then investigated in this work (Fig. 3). LMW colloidal carbohydrate amounts tended to decrease during the emersion period. This drop observed in winter was not significant ($p < 0.05$) but significantly visible in summer ($p < 0.01$). Besides, HMW colloidal carbohydrate amounts remained stable all over the emersion time, in winter or summer. These results were contradictory to other works which reported an increase of colloidal EPS in the early part of the tidal exposure and near to the end of the emersion (Taylor and Paterson, 1998). Nevertheless, Underwood and Paterson (2003) nuanced these results and argued that the evolution of colloidal EPS during the emersion period was often misinterpreted due to the extraction techniques used. The Dowex extraction that we performed allowed a fine separation between true colloidal and bound EPS surrounding diatom cells and/or entangled in the EPS matrix (Fig. 1). We already demonstrated that the use of this procedure in situ averted mixing a part of colloidal EPS with LMW bound EPS (Pierre et al., 2012). It can be estimated that the level patterns of colloidal EPS that we found in the present study were then closer to their function(s) and fate. Thus, many authors highlighted that colloidal EPS were C-sources in the trophic network of tidal mudflats, especially for heterotrophic bacteria and other micro-consumers (Hanlon et al., 2006; Pierre et al., 2012; van Duyl et al., 1999). However, the Dowex method used was derived from a published paper by Takahashi et al. (2009). In this work, the authors compared six EPS extraction methods tested on a diatom culture and proposed a same sort of comparison on sediments. Further experiments could be performed to access our statements about the Dowex method and confirm its high efficiency for in situ experiments.

On the other hand, the amounts of bound carbohydrates increased all over the tidal exposure. Even if this observation was not significant for W-LMW BC due to a lack of experimental values ($p < 0.05$), significant increases were observed in summer ($p < 0.01$). Owing to the use of the Dowex procedure, it was possible to distinguish the fate of true colloidal carbohydrates whose amounts dropped and bound carbohydrates whose concentrations increased during the emerged period. A

Table 3
Matrix of Pearson's correlation coefficients between carbohydrate contents of the different fractions (LMW/HMW: low and high molecular weight, CC: colloidal carbohydrates, BC: bound carbohydrates, RC: residual carbohydrates) and biotic/abiotic parameters measured on the sediment and during the two sampling periods (W: winter, S: summer).

a) Winter	W-LMW CC	W-HMW CC	W-LMW BC	W-HMW BC	Light	Salinity	Pore water	Bacterial abundance	Tidal coefficient	C:N
Chl *a*	−0.114	0.291	−0.087	0.007	0.257	0.417	0.556	−0.524	−0.391	nd
W-LMW CC	x	0.656*	0.852**	−0.185	0.698*	−0.387	0.083	−0.323	0.200	nd
W-HMW CC	x	x	0.795**	0.276	0.347	−0.393	−0.176	−0.126	0.427	nd
W-LMW BC	x	x	x	0.189	0.422	−0.733*	−0.241	−0.222	0.585	nd
W-HMW BC	x	x	x	x	−0.621	−0.272	−0.433	0.514	0.776**	nd
Light	x	x	x	x	x	0.097	0.522	−0.682*	−0.423	nd
Salinity	x	x	x	x	x	x	0.618	−0.008	−0.763*	nd
Pore water	x	x	x	x	x	x	x	−0.558	−0.654*	nd

b) Summer	S-LMW CC	S-HMW CC	S-LMW BC	S-HMW BC	Light	Salinity	Pore water	Bacterial abundance	Tidal coefficient	C:N
Chl *a*	0.409*	0.108	−0.122	−0.375*	0.037	0.306	0.600***	0.405*	0.163	0.086
S-LMW CC	x	−0.033	−0.062	0.097	0.027	0.061	0.362*	0.307	0.091	0.090
S-HMW CC	x	x	−0.576***	−0.304	0.288	0.120	0.293	−0.072	0.536***	−0.175
S-LMW BC	x	x	x	0.292	0.014	0.128	−0.338*	−0.136	−0.024	−0.046
S-HMW BC	x	x	x	x	−0.344*	−0.249	−0.090	−0.114	−0.267	0.163
Light	x	x	x	x	x	0.330*	−0.196	−0.033	0.507**	−0.231
Salinity	x	x	x	x	x	x	−0.421*	0.103	0.417*	0.066
Pore water	x	x	x	x	x	x	x	0.317	−0.009	−0.030

Values of r significant at *p < 0.05, **p < 0.01 and ***p < 0.001.
nd: non determined.

part of this result was already observed (Pierre et al., 2012), leaving us to suggest that bound fractions could be involved in the formation and adhesion of the micro-phytobenthic biofilm. The role of micro-phytobenthos as sediment stabilizers was well documented (Paterson and Black, 1999; Spears et al., 2008; Thornton et al., 2002; Underwood and Paterson, 1993; Widdows and Brinsley, 2002) and a reduction in erosion state was already correlated with increasing of carbohydrates concentrations (Sutherland et al., 1998). Numerous authors suspected the role of colloidal EPS in general on the sediment dynamics but not of a very specific and separate part of these exopolymers. Nevertheless, recent works brought colloidal and especially bound EPS to the fore, whose the production could be light-dependant (Giroldo et al., 2003; Hofmann et al., 2009; Pierre et al., 2010, 2012; Underwood and Paterson, 2003). Anyway, we noted that no light-dependant level was found for the bound fractions (Table 3).

Numerous authors try to understand how the composition and dynamic of EPS produced by benthic diatoms change depending on environmental conditions (Underwood and Paterson, 2003). Thus, biochemical nature, production pathways, role and fate of EPS in intertidal ecosystems are widely studied. In general, all authors point that chemical and physical properties of EPS produced in situ vary significantly in response to biotic and abiotic parameters. That is why important abiotic and biotic parameters were followed in this study, such as chl *a*, light, salinity, pore water amount, bacterial abundance, tidal coefficient, and C:N ratio, in view of understanding their involvement in EPS dynamic productions. We previously showed that salinity, pore water amount, light and chl *a* have to be considered when the seasonal dynamics of EPS are studied. However, it was also interesting to note that tidal coefficients could have a significant impact (Table 3) which was logical since tidal coefficients influence the degree of periodic tidal emersions and the light degree. Besides, no significant patterns were found for bacterial abundance or C:N ratios. It was not possible to correlate C:N ratios with the presence of marine bacteria/micro-algae, terrestrial or degraded organic matter.

3.2. Monosaccharide composition of EPS: roles and functions of the different fractions

Monosaccharide composition of EPS was investigated for colloidal, bound and residual fractions extracted in winter and summer (Fig. 4). The monosaccharide composition was heterogeneous depending on the concerned fractions and the low standard deviations (<5%) indicated that the types and quantities of EPS were finely controlled during the short-term emerged periods. Colloidal carbohydrates were rich in glucose (>50%), xylose, galacturonic acid and inositol, regardless of the sampling period. Indeed, colloidal carbohydrates are known as carbon trophic sources due to their high quantity of glucose (Abdullahi et al., 2006; Hofmann et al., 2009). Nevertheless, we noted that the colloidal carbohydrates collected in summer were richer in glucose than in winter (+4%, p value [5%] = 0.003). The same observation was also done for bound and residual fractions (+40 and 45% respectively). On the one hand, the high glucose ratio in residual fractions (22 to 42%) was not surprising since these samples correspond essentially to intracellular carbohydrates. The majority of the glucosyl units found in these fractions are components of storage polymers as chrysolaminaran (Underwood and Paterson, 2003). On the other hand, it seemed important to focus on the increase of glucose content in colloidal and bound fractions during summer. We suggested that the phenomenon was a seasonal response to environmental needs due to C-consumers, such as nematodes, hydrobia and herbivorous zooplankton, which are more abundant and more active in summer. Thus, colloidal carbohydrates dropped during the emersion time (Fig. 3) but glucose content were higher (Fig. 4). It is therefore possible that carbohydrate EPS are selectively used by heterotrophic bacteria depending on season. In this way, bacteria could use the different monosaccharides as substrate but with different degrees of induction. Besides, the ratios of structural sugars (xylose and mannose) and uronic acids (galacturonic acids) were lower in summer for colloidal and bound fractions.

Rhamnose was the monosaccharide widely found in LMW (>25%) and HMW (18 to 34%) bound carbohydrates in winter and summer. The high levels of deoxy sugars in this fraction were already observed in winter on the same sampling site (Pierre et al., 2012) and other studies (Hanlon et al., 2006). Owing to their surface-active properties, deoxy sugars can promote the biostabilization of sediments (Giroldo et al, 2003; Zhou et al, 1998) by facilitating the coagulation of particles and macromolecules (Khodse et al., 2007). Rhamnose could also play a role of biochemical sensor in micro-phytobenthic biofilms as a target of proteins involved in cell–cell transmission signals. However, the level of rhamnose was lower in summer for the HMW bound fractions, i.e. for carbohydrates closely attached to diatom cells. This observation suggested a potential lower biostabilization of the sediment in summer (LMW BC: 25.6%, HMW BC: 18%).

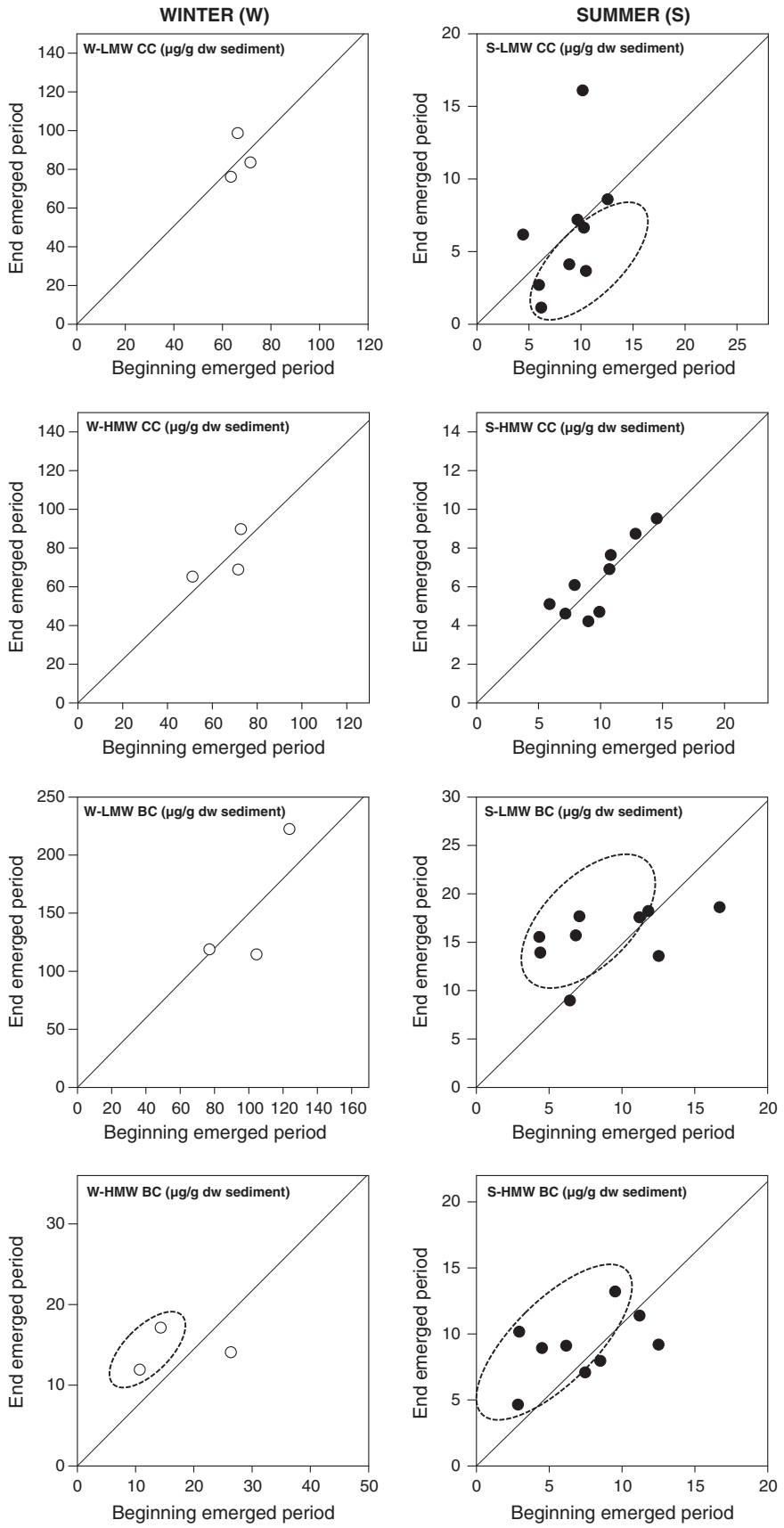

Fig. 3. Comparison of carbohydrate quantities (µg·g dry sediment^{-1}) during winter and summer of the extracted EPS fractions, at the beginning of the emerged periods with their values at the end of the emerged period. Abbreviations W, S, LMW, HMW, CC, BC and RC correspond respectively to winter, summer, low molecular weight, high molecular weight, colloidal carbohydrates, bound carbohydrates and residual carbohydrates. Regression lines are used to read the graphs. Data points in the upper left part correspond to variable which increased over the emerged period; data points in the lower right part correspond to variable which decreased during the same period. Data points close to the line: no change over the emerged period.

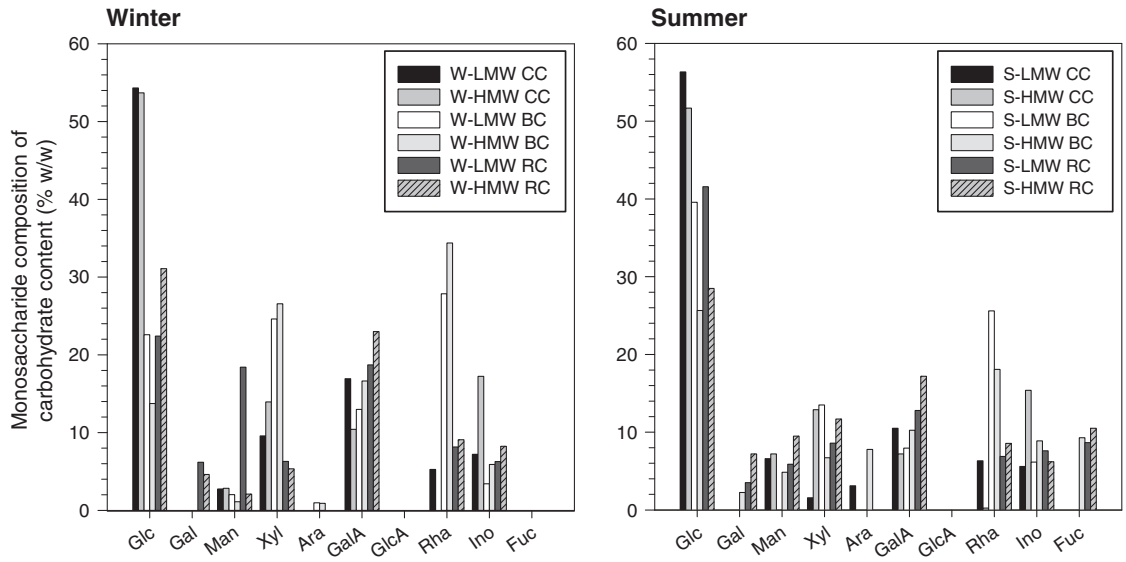

Fig. 4. Monosaccharide composition (% w/w) of the different fractions extracted in winter and summer. See Fig. 3 for abbreviations' meaning.

Fucose, a monosaccharide missing during winter samplings, was found in residual fractions (10%) collected in summer. We previously attributed the lack of fucose to the physiological state of the growing biofilm studied in winter (Pierre et al., 2012). During summer, colloidal and bound fractions did not contain fucose, in contrary to many works (Abdullahi et al., 2006; Hanlon et al., 2006; Hofmann et al., 2009; Takahashi et al., 2009). It is relevant to highlight that fucose is a monosaccharide involved in the metabolic pathways occurring during the degradation of β-1,3-glucan storage in diatom cells (Granum and Myklestad, 2001), which could explain its unique presence in residual fractions.

In the same way, another surprising sugar, inositol, was also found in the different samples and particularly in colloidal fractions (7 to 17%). As we already reported (Pierre et al., 2012), this monosaccharide could be a growth factor for heterotrophic micro-organisms involved in the micro-phytobenthic biofilm formation and synergistic relationships between benthic diatoms and bacteria (Lubarsky et al., 2010). Moreover, the levels seemed to be non-seasonal dependant (−5%).

A principal component analysis (PCA) along dominant sugar vectors was performed to clearly identify the differences in monosaccharide distributions of the EPS fractions (Fig. 5). Three distinct clusters were highlighted by ACP (C1 to C3). C1 was a cluster constituted of carbohydrates rich in glucose and inositol. Colloidal carbohydrates were sorted in C1. However, a significant seasonal difference was found between the monosaccharide distribution of W-LMW CC and S-LMW CC. Light and pore water could play a significant role in this dissimilarity (Table 3, $p < 0.05$). So, the monosaccharide distribution of LMW CC could be seasonal dependant (no cluster). These fractions are extremely labile and can be selectively consumed by heterotrophic bacteria. Cluster 2 represented fractions rich in rhamnose, xylose and galacturonic acid. Bound fractions, excepting S-HMW BC, belonged to C2. Previously, we noted that the rhamnose content was lower in S-HMW BC and we suggested that this ratio could play a major impact on sediment biostabilization.

It was noteworthy that residual carbohydrates were rich in glucose. However, galacturonic acid, galactose, fucose and mannose were ACP sugar vectors to take into account to sort these fractions (C3). Anyway, the results highlighted a strong heterogeneity of carbohydrate distributions in residual fractions which could be further investigated.

3.3. In situ modification of monosaccharides distribution: specific pathways?

The mechanisms allowing diatoms to rapidly change the EPS types being produced are not well understood (Hofmann et al., 2009; Underwood and Paterson, 2003). Moreover, the selective consumption of carbohydrate EPS by heterotrophic bacteria and the capacity of diatoms to change the composition of their EPS depending on environmental conditions are closely involved in these EPS changes (Giroldo et al., 2003). Previously, we showed that four major monosaccharides (glucose, xylose, galacturonic acid, and rhamnose) characterized the different fractions with some ratios above 20%. Fucose was only found during summer specifically in residual fractions. Moreover, these monosaccharides were representative sugar vectors in ACP analyses to identify clusters. That is why the standing stocks of these five monosaccharides were followed during the emerged periods in order to highlight possible specific pathways of selective consumption (Fig. 6). The first important observation to note was that the same monosaccharide distribution patterns were observed during winter and summer. Combined to our previous conclusions, this major result indicated that the distribution of monosaccharides in the fractions seemed to follow the same process regardless the season and also that the carbohydrate amounts, could vary in response to environmental parameters (cf. Section 3.2). Bacterial interactions, adhesion and biofilm formation, protection against salinity or migration into the sediment could be ecological and physicochemical phenomena involving EPS changes.

In details, glucose amounts stayed stable all over the emersion periods, despite a possible consumption of colloidal carbohydrates by heterotrophic bacteria. Galacturonic acid amounts dropped and rhamnose concentrations slightly increased for colloidal fractions extracted in winter and summer. The evolution in the galacturonic acid distribution was interesting and suggested that colloidal fractions were not only used as C-sources. Uronic acids are involved in several marine environmental processes including the production of macroaggregates, microbial adhesion and biofilm formation, binding of extracellular enzymes or ion sequestration (Bhaskar and Bhosle, 2005; Decho, 1990; Sutherland, 2001). This drop of uronic acids amounts could have an impact on the biofilm community, maybe by protecting diatoms from extreme salinity conditions.

If we focus on bound fractions, rhamnose amounts increased during the emersion time. We extensively discussed about the potential role of deoxy sugars in Section 3.2 (cell–cell interactions) and this observation seemed to confirm the involvement of rhamnose in adhesion phenomena or in the motility of diatom cells. Finally, residual fractions, rich in intracellular carbohydrates and refractory EPS were very complex in accordance with the literature. The important point to highlight was the slight drop of glucose amounts during the first hours of emersion, followed by a large increase of glucose ratios. This result could be due

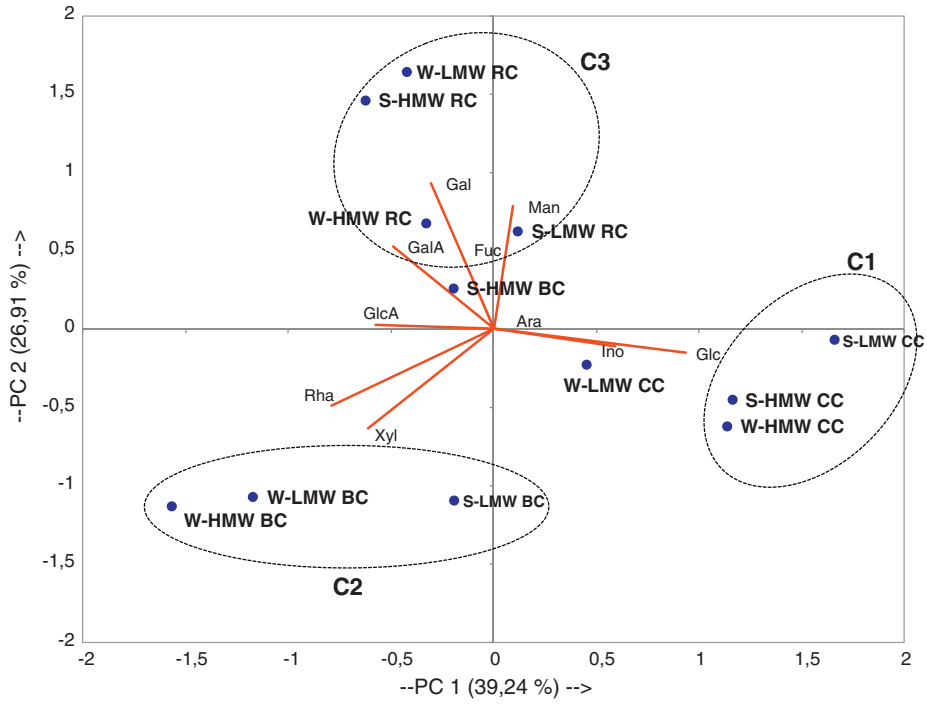

Fig. 5. Principal component analysis scatter plot grouping monosaccharide profiles of the different fractions along dominant sugar vectors. See Fig. 3 for abbreviations' meaning.

to the use of C-storage during the emerged period (metabolism) then to a drastic storing of carbon to survive during periods of darkness (Hanlon et al., 2006).

4. Conclusion

Based on extensive data set (>800 fractions), this paper focused on the seasonal dynamics of EPS and extensively carbohydrates produced on an intertidal mudflat during two different sampling periods (winter and summer). Firstly, colloidal carbohydrates, well known as C-sources for heterotrophic bacteria and other C-consumers, were steadily produced suggesting that an ecological equilibrium maybe existed between carbon production and consumption. The increase of bound carbohydrate levels during emersion times could show their involvement in adhesion/cohesive properties and/or in the locomotive properties of diatom cells which have to migrate back into the sediment at the end of emersion time. These dynamic pathways were identical between winter and summer, even if the data numbers allowed us to significantly

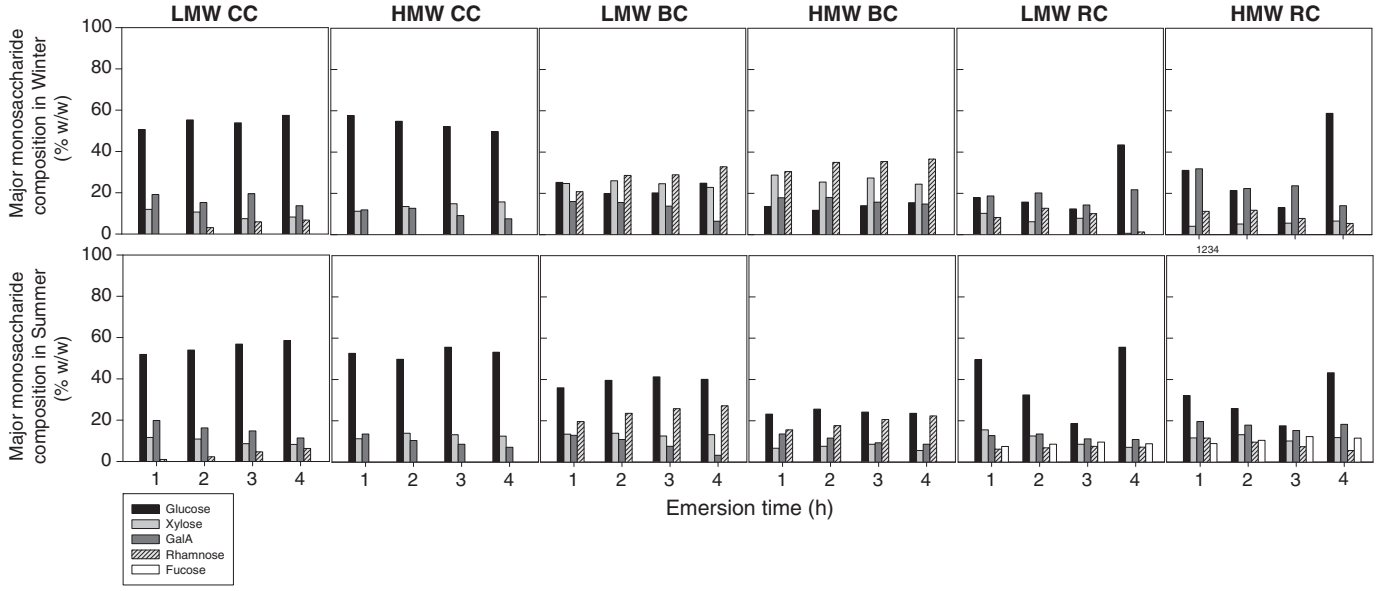

Fig. 6. Effect of the emersion time (average on the three sampling sites) on the major monosaccharide contents. Glucose, xylose, galacturonic acid, rhamnose and fucose are representative monosaccharides reported as characteristics of the physicochemical properties of the colloidal, bound and residual fractions. The variability within true sample replicates was less than 5%. See Fig. 3 for abbreviations' meaning.

confirm this hypothesis only for summer. The presence of proteins during summer was correlated to an additional C-storing for diatoms cells (residual fractions) and to a potential protection against high desiccation and salinity degrees. A number of abiotic/biotic parameters seemed to have impact on EPS dynamics and levels such as salinity, pore water amount, light and tidal coefficient. At last, GC/MS analyses showed that colloidal carbohydrates were glucose-rich, bound carbohydrates were significantly composed of rhamnose and residual fractions presented a wide variety of monosaccharides. All the fractions were richer in glucose during summer than winter, maybe to response to greater trophic needs. ACP analyses allowed refining the results and highlighted that colloidal carbohydrates formed a first cluster (glc and ino). Cluster 2 (xyl and rham) was constituted of bound carbohydrates and cluster 3 (fuc, galA, gal, and man) of residual carbohydrates. The evolution of five representative monosaccharides (glc, galA, xyl, rham, and fuc) was also followed in function of the tidal emersion periods and the results showed again that the distribution pathways did not change in winter or summer in contrary to monosaccharide amounts. We noted that the levels of galacturonic acid varied for colloidal and bound fractions, which could have an effect on binding forces, protection (via ion sequestration for instance) and cell–cell interactions in micro-phytobenthic biofilms. Besides, C-storage consumption and C-storing (glc, fuc, and man) were observed in residual fractions during the emerged periods. To conclude, many results were consistent with the literature and additional information was obtained concerning the influence of seasonal parameters on EPS levels, productions and dynamics. It should be interesting to check whether these conclusions could be observed on a much larger data set, specifically targeting light, salinity, pore water amount, and tidal coefficient as major parameters.

Acknowledgments

This study was supported by the Conseil Général of Charentes-Maritime through the Contrat de Projets Etat-Région “Littoral” and the Centre National de la Recherche Scientifique. The field sampling was supported by the French ANR (National Research Agency) through the VASIREMI project “Trophic significance of microbial biofilms in tidal flats” (contract ANR-06-BLAN-0393-01).

References

Abdullahi, A.S., Underwood, G.J.C., Gretz, M.R., 2006. Extracellular matrix assembly in diatoms (Bacillariophyceae). V. Environmental effects on polysaccharide synthesis in the model diatom, *Phaeodactylum tricornutum*. Journal of Phycology 42, 363–378.

Andersen, T.J., 2001. Seasonal variation in erodability of two temperate microtidal mudflats. Estuarine, Coastal and Shelf Science 49, 99–111.

Azerado, J., Henriques, M., Sillankorva, S., Oliveira, R., 2003. Extraction of exopolymers from biofilm: the protective effect of glutaraldehyde. Water Science and Technology 47, 175–179.

Bellinger, B.J., Abdullahi, A.S., Gretz, M.R., Underwood, G.J.C., 2005. Biofilm polymers: relationship between carbohydrate biopolymers from estuarine mudflats and unialgal cultures of benthic diatoms. Aquatic Microbial Ecology 38, 169–180.

Bhaskar, P.V., Bhosle, N.B., 2005. Microbial extracellular polymeric substances in marine biogeochemical processes. Current Science 88, 45–53.

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. Analytical Biochemistry 54, 484–489.

Christie, M.C., Dyer, K.R., Blanchard, G., Cramp, A., Mitchener, H.J., Paterson, D.M., 2000. Temporal and spatial distributions of moisture and organic content across a macrotidal mudflat. Continental Shelf Research 20, 1219–1242.

de Brouwer, J.F.C., Stal, L.J., 2001. Short-term dynamics in microphytobenthos distribution and associated extracellular carbohydrates in surface sediments of an intertidal mudflat. Marine Ecology Progress Series 218, 33–44.

de Brouwer, J.F.C., Stal, L.J., 2004. Does warm-water extraction of benthic diatoms yield extracellular polymeric substances or does it extract intracellular chrysolaminaran? European Journal of Phycology 39, 129–131.

Decho, A.W., 1990. Microbial exopolymer secretions in ocean environment: their role(s) in food webs and marine processes. Oceanography and Marine Biology. Annual Review 28, 73–153.

Decho, A.W., 2000. Microbial biofilms intertidal systems: an overview. Continental Shelf Research 20, 1257–1274.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350–356.

Falciatore, A., Bowler, C., 2002. Revealing the molecular secrets of marine diatoms. Annual Review of Plant Biology 53, 109–130.

Filisetti-Cozzi, T.M., Carpita, N.C., 1991. Measurement of uronic acids without interference from neutral sugars. Analytical Biochemistry 197, 157–162.

Frostick, L.E., McCave, I.N., 1979. Seasonal shifts of sediment within an estuary mediated by algal growth. Estuarine and Coastal Marine Science 9, 569–576.

Giroldo, D., Vieira, A.A.H., Paulsen, B.S., 2003. Relative increase of deoxy sugars during microbial degradation of an extracellular polysaccharide released by a tropical freshwater *Thalassiosira* sp. (Bacillariophyceae). Journal of Bacteriology 189, 7945–7947.

Granum, E., Myklestad, S.M., 2001. Mobilization of β-1,3-glucan and biosynthesis of amino acids induced by NH^{4+} addition to N-limited cells of the marine diatom *Skeletonema costatum* (Bacillariophyceae). Journal of Phycology 37, 772–782.

Hanlon, A.R.M., Belinger, B., Haynes, K., Xiao, G., Hofmann, T.A., Gretz, M.R., Ball, A.S., Osborn, M., Underwood, G.J.C., 2006. Dynamics of extracellular polymeric substances (EPS) production and loss in an estuarine, diatom-dominated, microbial biofilm over a tidal emersion–immersion period. Limnology and Oceanography 51, 79–93.

Haubois, A.G., Sylvestre, F., Guarini, J.M., Richard, P., Blanchard, G., 2005. Spatio-temporal structure of the epipelic diatom assemblage from an intertidal mudflat in Marennes–Oléron Bay, France. Estuarine, Coastal and Shelf Science 64, 385–394.

Hofmann, T., Hanlon, A.R.M., Taylor, J.D., Ball, A.S., Osborn, A.M., Underwood, G.J.C., 2009. Dynamics and compositional changes in extracellular carbohydrates in estuarine sediments during degradation. Marine Ecology Progress Series 379, 45–58.

Khodse, V.B., Fernandes, L., Gopalkrishna, V.V., Bhosle, N.B., Fernandes, S.G., Matondkar, S.G.P., Bhushan, R., 2007. Marine Chemistry 103, 327–346.

Lorenzen, S., 1966. A method for the continuous measurement of in vivo chlorophyll concentration. Deep-Sea Research 13, 223–227.

Lubarsky, H.V., Hubas, C., Chocholek, M., Larson, F., Manz, W., Paterson, D.M., Gerbersdorf, S.U., 2010. The stabilization potential of individual and mixed assemblages of natural bacteria and microalgae. PLoS One 5, e13794.

Paterson, D.M., Black, K.S., 1999. Water flow, sediment dynamics and benthic biology. Advances in Ecological Research 29, 155–193.

Pierre, G., Graber, M., Orvain, F., Dupuy, C., Maugard, T., 2010. Biochemical characterization of extracellular polymeric substances extracted from an intertidal mudflat using a cation exchange resin. Biochemical Systematics and Ecology 38, 917–923.

Pierre, G., Graber, M., Rafiliposon, B.A., Dupuy, C., Orvain, F., de Crignis, M., Maugard, T., 2012. Biochemical composition and changes of extracellular polysaccharides (ECPS) produced during microphytobenthic biofilm development (Marennes–Oléron, France). Microbial Ecology 63, 157–169.

Porter, K.G., Feig, Y.S., 1980. The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25, 943–948.

Serôdio, J., da Silva, J.M., Catarino, F., 1997. Non-destructive tracing of migratory rhythms of intertidal benthic microalgae using *in vivo* chlorophyll *a* fluorescence. Journal of Phycology 33, 542–553.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1987. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76–85.

Spears, B.M., Saunders, J.E., Davidson, I., Paterson, D.M., 2008. Microalgal sediment biostabilisation along a salinity gradient in the Eden Estuary, Scotland: unraveling a paradox. Marine and Freshwater Research 59, 313–321.

Staats, N., de Deckere, E.M.G.T., de Winder, B., Stal, L.J., 2001. Spatial patterns of benthic diatoms, carbohydrates and mud on a tidal flat in the Ems-Dollard estuary. Hydrobiological 448, 107–115.

Stal, L.J., Défarge, C., 2005. Structure and dynamics of exopolymers in an intertidal diatom biofilm. Geomicrobiology Journal 22, 341–352.

Sutherland, I.W., 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147, 3–9.

Sutherland, T.F., Grant, J., Amos, C.L., 1998. The effect of carbohydrate production by the diatom *Nitzschia curvilineata* on the erodability of sediment. Limnology and Oceanography 43, 65–72.

Takahashi, E., Ledauphin, J., Goux, D., Orvain, F., 2009. Optimizing extraction of extracellular polymeric substances (EPS) from benthic diatoms: comparison of the efficiency of six EPS extraction methods. Australian Journal of Marine and Freshwater Research 60, 1201–1210.

Taylor, I., Paterson, D.M., 1998. Microspatial variation in carbohydrate concentrations with depth in the upper millimeters of intertidal cohesive sediments. Estuarine, Coastal and Shelf Science 46, 359–370.

Thornton, D.C.O., Dong, L.F., Underwood, G.J.C., Newell, D.B., 2002. Factors affecting microphytobenthic biomass, species composition and production in the Colne Estuary (UK). Aquatic Microbial Ecology 27, 285–300.

Underwood, G.J.C., Kromkamp, J., 1999. Primary production by phytoplankton and microphytobenthos in estuaries. Advances in Ecological Research 29, 93–153.

Underwood, G.J.C., Paterson, D.M., 1993. Seasonal changes in diatom biomass, sediment stability and biogenic stabilization in the Severn estuary. Journal of the Marine Biological Association 73, 871–887.

Underwood, G.J.C., Paterson, D.M., 2003. The importance of extracellular carbohydrate production by marine epipelic diatoms. Advances in Botanical Research 40, 184–240.

Underwood, G.J.C., Paterson, D.M., Parkes, R.J., 1995. The measurement of microbial carbohydrate exopolymères from intertidal sediments. Limnology and Oceanography 40, 1243–1253.

Underwood, G.J.C., Boulcott, M., Raines, C.A., 2004. Environmental effects on exopolymeric production by marine benthic diatoms: dynamics, changes in composition, and pathways of production. Journal of Phycology 40, 293–304.

Underwood, G.J.C., Fietz, S., Papadimitriou, S., Thomas, D.N., Dieckmann, G.S., 2010. Distribution and composition of dissolved extracellular polymeric substances (EPS) in Antarctic sea ice. Marine Ecology Progress Series 404, 1–19.

van Duyl, F.C., de Winder, B., Kop, A.J., Wollenzien, U., 1999. Tidal coupling between carbohydrate concentrations and bacterial activities in diatom-inhabited intertidal mudflats. Marine Ecology Progress Series 191, 19–32.

Widdows, J., Brinsley, M., 2002. Impact of biotic and abiotic processes on sediment dynamics and the consequences to the structure and functioning of the intertidal zone. Journal of Sea Research 48, 143–156.

Wimpenny, J., Manz, W., Szewzyk, U., 2000. Heterogeneity in biofilms. FEMS Microbiology Review 24, 661–671.

Wingender, J., Neu, T.R., Flemming, H.C., 1999. What are bacterial extracellular polymeric substances? In: Wingender, J., Neu, T.R., Stadler, P. (Eds.), Microbial Extracellular Polymeric Substances: Characterization, Structure and Function. Springer-Verlag, Berlin, pp. 1–15.

Yallop, M.L., Paterson, D.M., Wellsbury, P., 2000. Inter-relationships between rates of microbial production, exopolymer production, microbial biomass and sediment stability in biofilms of intertidal sediments. Microbial Ecology 39, 116–127.

Zhou, J., Mopper, K., Passow, U., 1998. The role of surface-active carbohydrates in the formation of transparent exopolymer particles by bubble adsorption of seawater. Limnology and Oceanography 43, 1860–1871.