Biochemical composition and changes of extracellular polysaccharides (ECPS) produced during microphytobenthic biofilm development (Marennes-Oléron, France).

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Abstract

The main goal of this work was to study the dynamics and biochemical composition of ExtraCellular Polysaccharides (ECPS), a fraction of the Extracellular Polymeric Substances (EPS) produced during the development of a microphytobenthic biofilm in a European intertidal mudflat (Marennes-Oléron Bay, France) during winter. Microphytobenthic biomass was surveyed during four consecutive emersion periods to confirm the biofilm growth. Bacteria abundance was also checked considering the importance of heterotrophic bacteria observed by various authors in the dynamics of EPS. Various colorimetric assays, coupled to biochemical chromatographic analysis were used to characterize the three main fractions of extracted EPS: colloidal, bound and residual. The monosaccharide distribution of colloidal ECPS highlighted their role of carbon source for bacteria (> 50 % of glucose) even if no increase of colloidal carbohydrate amounts was observed during the tidal exposure. Bound

ECPS were composed of deoxy or specific sugars (30 % rhamnose) and uronic acids (18 % galacturonic acid). Their levels and dynamics could be correlated to the development of the microphytobenthic biofilm, enhancing the stabilization of the sediment or increasing binding forces accordingly. Residual fractions, containing refractory bound ECPS and other internal polymeric substances, were composed of various carbohydrates. The high ratio of glucose in these fractions (18 to 43 %) was interesting as it was once attributed to colloidal sugars due to poor extraction procedures. Finally, the presence of inositol (15 %) was significant since no author has highlighted it before, knowing that inositol is a major growth factor for heterotrophic bacteria.

Key words: extracellular compounds, EPS, biochemical characterization, biofilm, benthic ecology, intertidal mudflat

Introduction

Tidal flats are an essential system within the trophic network and contribute greatly to the productivity of coastal ecosystems. The high primary and secondary productivity of mudflats is linked to physical and biological processes involving unicellular micro-organisms. The development of conceptual models for these highlighted the role of diatoms [16] and bacteria in the sediment mudflats [21]. More generally, regarding biomass and production, it is known that microphytobenthos are a major component of these intertidal sediment communities [38, 39, 41].

Diatoms have been described in several recent studies showing that they were closely involved in the formation of biofilms [11, 27]. They are essential for the health of the marine environment through the excretion of large quantities of Extracellular Polymeric Substances (EPS), which are involved in particular in their motility systems. In intertidal benthic

ecosystems, this motility is essential to enable epipelagic diatoms to migrate into the photic zone of the sediment during the time of emersion [39]. In addition, previous research has shown that EPS can be used as a carbon source by the bacterial community and the macrofauna [17]. EPS also affect the microenvironment of biofilms by varying physico-chemical parameters like porosity, density, sorption properties, hydrophobicity and mechanical stability [13, 14]. Thus, the composition of EPS is clearly linked to the erosion of sediments, either increasing their stability [26] or decreasing stability in case of nutrient stress during growth stationary phase [25].

The microbenthic community is mostly composed of water, diatoms, bacteria, eukaryotic microbes, EPS and inorganic particles. According to Wingender et al. (1999), the EPS matrix is composed of a wide variety of proteins, polysaccharides (ECPS), lipids and other more surprising compounds such as nucleic acids. This very complex composition makes biochemical analysis particularly difficult due to the diversity in sugar monomers, linkages, low concentrations and the interactions of compounds during assays. Besides, the extraction of EPS is another challenge since the polymers are highly complex and exist in a continuum from tightly cell bound to loosely cell associated. The extraction and separation methods have to be selected in term of the compounds to be extracted while controlling what happens to other compounds; minimal cell lysis and no disruption or alteration of the EPS are basic requirements [9]. In this way, Takahashi et al. (2009) clearly demonstrated that extraction of colloidal and bound EPS with a cation exchange resin was the best method to obtain high yield of carbohydrates and a proper extraction without causing cell disruption or contamination by internal storage materials.

Extraction protocols can distinguish a range of EPS types, depending on the nature of the extraction and the location of EPS in the sediment [6] such as: (i) colloidal fractions corresponding to the polymeric substances excreted in the medium, (ii) bound fractions which

are the compounds surrounding the cells (that should be associated to cell adhesion and/or protection) and (iii) residual fractions, corresponding to the internal polymers and refractory bounds EPS [37]. It has been demonstrated that diatom and microbial EPS can be also separated into two major types: Low Molecular Weight (LMW) and High Molecular Weight (HMW).

The present investigation used *in situ* an improved extraction method involving a cation exchange resin to better characterize the EPS and particularly ECPS secreted by a microphytobenthic biofilm [37]. The three fractions of EPS were extracted from intertidal sediments (Marennes-Oléron Bay, France), during the development of diatom-dominated biofilm [18, 19, 25] and three consecutive diurnal emersion periods during winter. The main goal of the study was to determine and understand, through biochemical analysis of these fractions, the dynamics of ECPS during a microphytobenthic biofilm growth.

Materials and methods

Intertidal mudflat samples

The mudflat samples used in this study were collected from Marennes-Oléron Bay (Atlantic Coast of France), during one week in February 2008 (winter) at low tide (Fig. 1). The field sampling was organized as a chessboard where we defined square sampling units (2m-side) separated by alleys (2m in width). Every day, 3 squares were randomly taken to account for spatial heterogeneity. Three cores of each selected square were sampled every hour during the emersion period, for 3 consecutive days. Sediment samples from each square were collected by using core diameter of 20 cm. For each core, the top 1cm was collected three times and pooled. After each sampling, sediment was brought back from the field by using a watercraft for an immediate EPS extraction on fresh sediments on the upper shore. Biochemical analyses were performed in triplicate on the colloidal, bound and residual fractions (216 fractions).

97 *Environmental parameters*

98 Light was measured using a Li-Cor sensor which was recorded every minute during sampling
99 days. Enumeration of bacteria: to separate bacteria from sediment particles, incubation in
100 pyrophosphate (0.01 M for at least 30 min) and sonication (60 W) were performed. Bacteria
101 from sediment were labeled using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)
102 ($2500 \mu\text{g} \cdot \text{l}^{-1}$), filtered onto 0.2 μm Nucleopore black filter [30] and then counted by
103 microscopy (x 1000, Axioskop, Zeiss). The chlorophyll *a* concentration in the sediment was
104 measured using fluorometry method [23] on two types of samples which were obtained from
105 the top 0.2 cm (more representative of the chlorophyll *a* produced by diatoms) or 1 cm of
106 sediment cores. Initially, 16 measures of chl *a* concentration were planned but only 9
107 measures were done in the top 1 cm of sediment cores (from the day 2/18/08 to the day
108 2/20/08) and 12 measures were done in the top 0.2 cm (from the day 2/18/08 to the day
109 2/21/08). Missing data were due to experiment constraints.

110 *Materials*

111 Dowex Marathon C, bicinchoninic acid (BCA) Protein Assay Kit, Azure A, N,O-
112 bis(trimethylsilyl)trifluoroacetamide: trimethylchlorosilane (BSTFA: TMCS) (99:1) were
113 obtained from Sigma-Aldrich. Standard carbohydrates (dextran, dextran sulfate, heparin,
114 fucoidan, glucose, galactose, rhamnose, fucose, fructose, xylose, arabinose, mannose, *myo*-
115 inositol, glucuronic and galacturonic acid) and a protein standard (Bovine Serum Albumin,
116 BSA) were obtained from Sigma-Aldrich. The DB-1701 JandW Scientific column (30 m, 0.32
117 mm, 1 μm) for Gas Chromatography-Mass Spectrometry analysis (GC/MS) was obtained
118 from Agilent.

119 *EPS Extraction*

The extraction method [37] was done immediately after sampling and sediment mixing in the field. 20 mL of fresh mudflat was continuously mixed with 20 mL of Artificial Sea Water (ASW 30 Practical Salinity Units) during 1 h in darkness at 4 °C and then centrifuged at 3500 g and 4 °C for 10 min. The supernatant (a) containing colloidal EPS was collected and stored at 4 °C. 20 mL of ASW and 1 g of activated Dowex (Marathon C, activated in Phosphate Buffer Saline for 1 h in the dark) was added to the sediment pellet (b). 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 (c) and a cap containing intracellular and residual polymers (d) were obtained. The cap was then frozen. The residual polymers were extracted from the frozen sediment samples (d), 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 (a) 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 (Low Molecular Weight, LMW fraction) and a precipitate pellet (High Molecular Weight, HMW fraction). Finally, the fractions were dried under air flow and stored at -20 °C.

Biochemical analysis of EPS and carbohydrate fractions

Total sugar content was determined using the phenol-sulfuric acid assay, using glucose as a standard [10]. Briefly, 1 mL of 5 % phenol and 5 mL sulfuric acid were added to 1 mL EPS solution, previously resuspended in ultra-pure water, and vortexed. Measurements were read after 30 min with a spectrophotometer at 485 nm. Total sugar amounts for the fractions were measured and normalized to chlorophyll *a* (chl *a*). This normalization is classically used in the

literature and allows to overestimates diatom EPS and ECPS production, comparing to other EPS sources, especially when the mudflat is mainly composed of microphytobenthos [18].

Protein content was determined using the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) as a standard [31]. Briefly, 0.2 mL EPS, previously resuspended in ultra-pure water, were added to 2 mL BCA active reagent, and gently vortexed. Measurements were read after 15 min of heating at 60 °C, with a spectrophotometer at 562 nm.

Uronic acid content was determined using the meta-hydroxydiphenyl method (MHDP), using galacturonic and glucuronic acids as standards [3, 12]. Briefly, 40 µL of 4 M sulfamic acid and 2.4 mL of 0.075 M sodium tetraborate were added to 400 µL of EPS, previously resuspended in ultra-pure water, and gently vortexed. 80 µL of 0.15 % MHDP were added to the solution after 20 min of heating at 80 °C. Sample was strongly vortexed and allowed to rest 10 min. Measurements were read with a spectrophotometer at 525 nm.

The sulfate content was measured by the Azure A [22] and the Ba/Cl₂ gelation method [7], using Dextran sulfate as a standard. Concerning the Azure A assay, 2 mL of 10 mg/L Azure A were added to 200 µL EPS, previously resuspended in ultra-pure water, and gently vortexed. Measurements were read with a spectrophotometer at 535 nm. Concerning the Ba/Cl₂ gelation assay, 10 mg EPS were hydrolyzed in 0.5 mL of 2 M HCl for 2 h at 100 °C. Ultrapure water was added (qsp 10 mL) and the sample was then centrifuged for 10 min at 5000 g. For the turbidimetric assay, 1 mL supernatant, 9 mL ultra-pure water, 1 mL of 0.5 N HCl and 0.5 mL of Ba/Cl₂ – gelatin reagent were added in a glass tube. After 30 min resting at room temperature, measurements were read with a spectrophotometer at 500 nm.

Characterization of carbohydrate fractions by GC/MS

Prior to carbohydrate characterization by GC/MS, EPS fractions were solubilized in 5 mL of ultrapure water, dialyzed (6-8 KDa) and freeze-dried [2]. EPS were then dissolved in 2 M HCl at 50 mg/mL and heated at 90 °C for 4 h. The preparation (which contained mostly ECPS monomers) was then freeze-dried and stored at -20 °C. Analysis of the carbohydrate fractions 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&W Scientific column (30 m, 0.32 mm, 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 [29]. Each monosaccharide amount measured by GC/MS was expressed depending on the total carbohydrate amounts measured by biochemical assays, while considering the sampling date and the emersion time.

Statistical analysis

One-way analysis of variance (ANOVA), was used to analyze changes in carbohydrate and uronic acid amounts among abiotic parameters (sampling location, emersion time) for each day. Data transformations (root) were performed each time it was required to check application conditions (normality). Normality tests of residuals were tested by Kolmogorov-Smirnov test and homoscedasticity by Bartlett test. In addition, post hoc procedures (Tukey test) were performed to analyze pairwise differences. Pearson correlations were performed to investigate the relationships between the different EPS fractions with biotic parameters (bacterial density, chl *a* biomass) and also abiotic parameters (luminosity). Statistical analyses were run using the statistical software XLStat (Addinsoft).

Results

Environmental parameters

Light measurements showed the higher brightness during the first day of sampling ($1000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) (Fig. 2A). The most important brightness peaks were obtained for the other days around 12-14h (600 to $800 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The comparison between ambient light climate and carbohydrate concentrations did not reveal a specific impact of light on the dynamics of ECPS except for bound HMW ($r = -0.444$, $R^2 = 0.197$). The measures of chlorophyll *a* remained constant during the sampling period, with values around $21.5 \mu\text{g/g}$ of dry sediment (Fig. 2B). The survey showed the presence of a biofilm and its formation (Fig. 2C). A significant increase of the Chl *a* concentration was observed for the day 2/21/08 (P value [5 %] = 0.003) (Fig. 2C).

Carbohydrate content

ECPS amounts and their dynamics for the three fractions are presented in Fig. 3, depending on the emersion time and the sampling day. The quantities of total colloidal carbohydrates (LMW and HMW) were close to $6.5 \mu\text{g sugar} \cdot \mu\text{g chl } a^{-1}$ (Fig. 3A). The results showed that the amounts of LMW total sugars remained constant around $3.5 \mu\text{g sugar} \cdot \mu\text{g chl } a^{-1}$ during the sampling campaign (except for 2/20/08-4h, with a concentration close to $5.5 \mu\text{g sugar} \cdot \mu\text{g chl } a^{-1}$). HMW total sugar quantities were slightly lower ($3 \mu\text{g sugar} \cdot \mu\text{g chl } a^{-1}$ on average, with an increase for 2/20/08-4h to $5 \mu\text{g sugar} \cdot \mu\text{g chl } a^{-1}$). Differences in concentrations related to the sampling squares were found but were not significant (p values [5 %] = 0.970 and 0.121 for HMW and LMW colloidal carbohydrates respectively). No significant effect of the sampling day was observed on the dynamic of LMW and HMW colloidal ECPS (p value [5 %] = 0.447 and 0.471). Statistical analysis showed that the dynamics of LMW and HMW bound carbohydrates varied with the sampling day (p value [5 %] = 0.001 and 0.023

respectively) (Fig. 3B). Besides, no significant effect of the spatial heterogeneity was found for LMW and HMW bound carbohydrate concentrations (p values [5 %] = 0.957 and 0.324 respectively). The LMW total sugar quantities in bound fractions tended to increase during the emerged phase. This trend was especially pronounced for the period 2/20/08 1 h - 4 h since the quantity nearly doubled (6 μg to 13 μg sugar . $\mu\text{g chl } a^{-1}$) (p value for day 2/20/08 [5 %] = 0.028). Concerning the residual fractions (Fig. 3C), there was a good level of repeatability for the LMW carbohydrate amounts between the sampling squares since no significant difference was found (p value [5 %] = 0.556). It is difficult to highlight a particular trend, except a stagnation of LMW carbohydrate amounts (around 20 μg sugar . $\mu\text{g chl } a^{-1}$). The amounts of HMW carbohydrate quantities in the residual fractions were found to increase but not significantly during the emersion of the sediment (p values for days 2/18-19-20/08 [5 %] = 0.576, 0.976 and 0.057 respectively), at higher concentrations (30 to 75 μg sugar . $\mu\text{g chl } a^{-1}$).

The change in distribution of total carbohydrates between LMW and HMW for the colloidal, bound and residual fractions is presented in Fig. 4. The LMW colloidal carbohydrates were slightly and significantly more abundant than the HMW colloidal carbohydrates (p value [5 %] = 0.003). For the bound fractions, the carbohydrates were produced in very precise quantities as indicated by the low standard deviation between the different sampling sites. The bound carbohydrates seem to be produced at a constant and significant ratio of 90/10: LMW/HMW (p value [5 %] < 0.0001), compared with 55/45 for the colloidal fractions. The residual fractions were essentially composed of HMW compounds, *i.e.* 60-70 % for total carbohydrates.

Sulfate and protein contents

No sulfated groups were detected in the colloidal and bound fractions. Very low concentrations of proteins were found in the bound fractions. However, the BCA method used for analysis was not enough accurate, so these concentrations were considered close to zero. Sulfated components were detected in the residual fractions (Table 1). These amounts were relatively constant. However, these data should be interpreted with precaution in view of the significant deviations obtained between the different sampling sites. Protein concentrations were also measured in the residual fractions (Table 1). The quantities of proteins remained constant around $20 \mu\text{g} \cdot \mu\text{g chl } a^{-1}$ throughout the emersion.

Changes in ECPS composition

The changes in ECPS composition were carried out by GC/MS analysis (Fig. 5). The LMW colloidal fractions (Fig. 5A) contained about ± 23 % uronic acids (80 % galacturonic acid and 20 % glucuronic acid). These levels confirmed the results obtained by colorimetric assays. The LMW colloidal fractions contained more than 50 % glucose but also 5 % rhamnose, 10 % xylose and 7 % inositol (as myo-inositol). The percentage of rhamnose increased during the emerged periods (0 to 6.75 %). In contrast, the percentage of mannose and xylose decreased slightly during the four hours of emersion (12 % to 8.25 % and 5 % to 1 % respectively). For the HMW colloidal fractions, the percentage of uronic acids was lower than in the LMW fractions and decreased during the emerged periods (12 % to 7.7 %). The HMW colloidal fractions did not contain rhamnose. Concerning the monosaccharide composition of the bound fractions, one example of a spectrum analysis is presented in Fig. 6. The LMW bound fractions (Fig. 5B) were rich in rhamnose (20.8 % to 32.9 % during the emerged periods), xylose (± 24 %), glucose (± 21 %) and galacturonic acid. Small amounts of arabinose and mannose were also detected in the LMW bound fractions. The level of rhamnose greatly increased during the emerged periods. In contrast, the percentage of galacturonic acid ratios decreased significantly between the first and last hour of emersion (16 % to 6.5 %). For the

HMW fractions, rhamnose represented about 35 % of the total carbohydrates. The percentage of xylose was similar in LMW and HMW bound fractions (± 25 %). The percentage of galacturonic acid remained constant in HMW fractions at around 16 % during the emersion. The monosaccharide composition of residual fractions was different compared to colloidal and bound fractions (Fig. 5C). The concentration of each monosaccharide varied widely during the emerged periods, making it difficult to define a typical composition. For the LMW fractions, the amount of rhamnose was relatively constant during the first hours of emersion (± 10 %) and decreased drastically to 1 % during the last hour. A similar observation can be made for the percentage of xylose in these fractions (10 % to 0.7 %). In addition, the percentage of glucose followed an amazing trend during the emerged periods, decreasing slightly during the first three hours of emersion (18 % to 12 %) and dramatically increasing during the last hour to a level close to 43 % or almost half of the total monosaccharides. Similar observations were made for the HMW fractions, particularly regarding the changing levels of rhamnose (11 to 5 %) and glucose (31 to 13 % during the first three hours then an increase to 59 % during the fourth hour). Finally, the increase in the levels of unknown peaks suggests that the LMW and HMW residual fractions are really complex.

ECPS dynamics and bacteria abundance

The sediment was more rich in bacteria during the day 2/19/08 (around $5e^{+9}$ cells . g⁻¹ dry sediment) (Fig. 2B). The day 2/18/08 showed the lowest concentrations of bacteria ($1e^{+9}$ cells . g⁻¹ dry sediment). A decrease in the amount of bacteria was observed during the 2/20/08 ($5e^{+9}$ to $2.5e^{+9}$ cells . g⁻¹ dry sediment), with significant differences found for the bacterial densities at 0h, 1h and 3h of emersion (P value [5 %] = 0.013). During this period, a strong increase in bound LMW carbohydrates in the sediment was observed (Fig. 2B). Nevertheless, no significant correlation was found between bacteria and bound LMW carbohydrates ($r = -$

0.21, $R^2 = 0.044$), bound HMW carbohydrates ($r = 0.186$, $R^2 = 0.035$) and more generally bound (LMW + HMW) carbohydrates ($r = -0.17$, $R^2 = 0.029$).

Discussion

Methodological limitation

Overall, 1g of dry sediment was composed of about 1600 to 1900 μg of total carbohydrates and about 300 to 600 μg of total proteins, without normalization to Chl *a*. Although a large portion was unknown, authors have considered that natural mudflats samples may contain large quantities of impurities [8, 40] and it has been proved that the phenol sulfuric acid assay was not 100 % efficient for measuring complex polysaccharides, causing an underestimation of the total quantities of EPS [21]. Previous results obtained at the same station in 1998 showed that 1 g of sediment contained 700 to 1100 μg of carbohydrates [35]. These authors have worked on the first 5mm of sediment, which represent more accurately the microphytobenthos biofilm. Our “first 10 mm approach” could overestimate diatom EPS production due to the contamination from other sediment EPS sources [28].

ECPS dynamics during the biofilm development process

The ECPS dynamics in relation to the microphytobenthic biofilm development were investigated. A microphytobenthic biofilm development was confirmed (Fig. 2B, 2C) during the sampling period and study conditions (Fig. 2A). One main trend of constitution was observed during the biofilm development process.

The rate of EPS production by diatom biofilms during tidal exposure periods is affected by endogenous rhythms, light levels and nutrient status [17, 40]. Given the current literature, numerous authors explained the need to understand the biochemistry and physical nature of EPS to link their carbohydrate composition with their ability to become an integral part of the

biofilm matrix owing to their physico-chemical properties [1, 4]. Colloidal carbohydrates were consistently produced during the three days of analysis (Fig. 3A). No particular trend was found during the emerged periods, in contrast to the results obtained by Taylor et al. (1999) which showed an increase in colloidal carbohydrate levels towards the end of the emerged periods. It is noteworthy that Taylor worked on EPS fractions containing colloidal EPS and internal polymers. LMW bound carbohydrate amounts strongly increased towards the end of the emerged period (Fig. 3B) in contrast to HMW bound carbohydrates. Many authors showed an increase in bound carbohydrate amounts during the tidal exposure period [17, 32, 38]. Bound fractions were composed of 90 % LMW carbohydrates and this percentage was extremely constant throughout the sampling period (Fig. 5B). This high ratio of LMW carbohydrates could be attributed to the acidic properties of the Dowex which could hydrolyze a part of HMW ECPS, thus producing an overestimation of LMW carbohydrates. Cell-cell and cell-substratum interactions are facilitated by the production of tightly bound carbohydrates [4, 42] through the presence of uronic acids (Table 1). The anionic properties of bound ECPS, conferred by uronic acids, could allow association of divalent cations, providing greater binding forces during the biofilm development process [36].

Residual fractions, composed of refractory bound ECPS, internal polymers and all sort of sources of ECPS, were rich in carbohydrates, exceeding six or seven times the colloidal and bound carbohydrate quantities (Fig. 3C). In contrast with other works, the abundance in carbohydrates did not increase during the daytime emersion period. Indeed, photoassimilated carbons, attributed to glucan and/or chrysolaminaran, could be stored in diatoms to provide energy during periods of darkness [39]. Otherwise, the results showed the presence of proteins in residual fractions (Table 1) which could be use for additional C-stores and/or structuring microalgae cells (glycoproteins) [5, 37].

Changes in ECPS composition and ecological relevance

Considering the low standard deviations found between our true replicates ($< 5\%$), it can be considered that the type of monosaccharides, their turnover, and their amounts were controlled during short-term emerged periods by complex systems involving environmental conditions, sediment erosion or the transport and deposition cycle [40]. The monosaccharide composition was heterogeneous between the different EPS fractions (Fig. 5) [34]. Colloidal ECPS were mainly composed of glucose, galacturonic acid, xylose and inositol (Fig. 5A). Bound EPS had a more balanced distribution of monosaccharide components: rhamnose, xylose, glucose and galacturonic acid (Fig. 5B). Residual fractions were composed of glucose, mannose, rhamnose, galactose and galacturonic acid (Fig. 5C). The changes in EPS composition during the biofilm development process were compared to previous works which classically extracted multiple EPS: (colloidal EPS, colloidal extract (cEPS), EPS from hot water extraction (HW) and EPS from hot bicarbonate extraction (HB)). The results were then adapted to the conceptual model of Underwood and Paterson (2003) and a discussion was proposed, based on Fig. 7.

At this point, it is noteworthy to declare that it was surprising that fucose, a recurrent deoxy sugar found in the literature, has not been highlighted although the GC-MS method allowed its detection. It was possible that the lack of fucose was linked to the environmental conditions, the physiological state of the microphytobenthic biofilm and the period of the sampling campaign (winter).

The becoming of colloidal ECPS: are they only used as a C-source for bacteria?

LMW colloidal fractions could be compared to the low molecular weight exudates of Underwood and Paterson (2003), the colloidal fractions of Bellinger et al. (2009) or Hofmann et al. (2009). These exudates were mainly composed of glucose (53 %). HMW colloidal fractions (Fig. 7) could be compared to the category of colloidal EPS [40] or cEPS of

Abdullahi et al. (2006) and Hofmann et al. (2009). Carbohydrate content was mainly composed of glucose (55 %) (Fig. 5). Numerous studies have highlighted the role of bacteria in the dynamics of EPS on sediments [17, 21]. To better understand colloidal EPS and ECPS dynamics during the biofilm development process, it is also important to focus on these bacteria. Literature showed that microorganisms were able to produce specific bacterial EPS and to consume polymeric substances, as ECPS, for their own development. In this way, recent studies showed a decrease of LMW and HMW colloidal carbohydrates due to bacterial consumptions [21]. The present investigation did not show such a decrease (Fig. 2B) even if our specific sugar distribution of LMW colloidal fractions showed amounts of monomers close to those obtained by many authors, with the prevalence of glucose (Fig. 5). It is noteworthy that other monosaccharides could be involved in this consumption phenomenon. The percentages of hexoses (xylose, mannose) decreased during the emerged periods, suggesting that they were may be more easily assimilated or used by heterotrophic bacteria. Nonetheless, the important levels of xylose and mannose could be due to physiological processes to enhance the assimilation of specific proteins or molecules in cells [20]. The composition of colloidal ECPS could suggest that they were not only used as a nutrient source. In this way, the high level of galacturonic acid (> 12 %) and its decrease during the biofilm development was intriguing. Colloidal uronic acids could be involved in the formation and maintenance of the microphytobenthic biofilm, especially at the end of emersion time, through ion sequestration, negative charge, ECPS solubility, hydrophobicity [36]. Unfortunately, statistic tests failed to demonstrate a significant effect of the emersion time on uronic acid dynamics.

Besides, the presence of inositol (8 – 15 %) was also interesting, given that no author has highlighted it. This molecule plays different and important roles as structural basis for a number of secondary messengers in eukaryotic cells and mostly as a major growth factor for

heterotrophic bacteria, implying that this sugar could be produced in the sediment, maybe by diatoms, to promote the symbiotic phenomenon led to the formation of benthic biofilm.

Bound ECPS, the active players involved in the biofilm construction

LMW bound fractions (Fig. 7) were obtained for a first time properly, without contamination by residual polymers since less glucose was found in these fractions, comparing to HMW fractions of Hanlon et al., (2006). Rhamnose (25 %), xylose (22 %), glucose (20 %) and galacturonic acid (13 %) were the main carbohydrates. HMW bound fractions were very similar to LMW and were mainly composed of rhamnose (30 %), xylose (28 %), galacturonic acid (18 %) and glucose (13 %). The Dowex-method allowed highlighting the presence of important levels of deoxy sugars in the fractions. Indeed, the percentage of rhamnose strongly increased during the emerged period, in contrast to the percentage of galacturonic acid, which decreased, and both xylose and glucose, which remained constant. Deoxy sugars, as rhamnose, can promote biostabilisation of sediments [15, 43] owing to their surface active properties. They can also influence the hydrophobic character of ECPS, by enhancing their adhesion to the sediment or by regulating salinity and desiccation phenomena through different retention rate of water at the surficial sediments [33]. Rhamnose is also a component of the outer cell membrane of certain bacteria and can be specifically recognized by lectins, transmitting signals to the cell- interior and conferring specific properties. Given some of these properties, the increase in the percentage of rhamnose in this fraction could be linked to the role of these bound ECPS, for example improving cell-cell interactions between bacteria and diatoms. Thus, bound carbohydrates could be closely involved into the formation of the extracellular matrix of the microphytobenthic biofilm and also into its biochemical short-term modifications in response to environmental conditions. Alternatively, rhamnose could play a role as a biochemical sensor to control “the biofilm entity”.

The amount of bacteria measured on the surface of the sediment seemed to provide another possible role of bound EPS. The results appeared to show a relationship between bacteria abundance and bound carbohydrate dynamics (Fig. 2B, Fig. 3B). The results suggested a potential negative effect of bound EPS on bacterial division, but no significant correlation was found between bacteria and bound carbohydrates.

Residual fractions and C-storage

LMW and HMW residual fractions (Fig. 7) could correspond to intracellular and cell wall associated polymers but also consist of a mixture of different refractory EPS not totally extracted by the Dowex-method. Fractions could be compared to a part of HW and all HB fractions of Abdullahi et al. (2006) or Hanlon et al. (2006). The carbohydrate content was composed of glucose (18 to 43 %), galacturonic acid (19 %), mannose (18 %), xylose (10 %) and rhamnose (10 %). The observations of many authors indicated that, these fractions were very complex, due to the presence of proteins and sulfated groups, and the different monosaccharides detected could be involved in the formation of structural polymers, glycolipids or proteoglycans [24]. The important point to highlight was the slight decrease in the percentage of glucose during the first hours of emersion. However, towards the end of the emerged periods, the quantity of glucose doubled or tripled. This observation could be correlated to the capacity of diatoms “to sense the end of emersion”, by storing a maximum amount of carbon for their metabolism to survive during periods of darkness. This result was partly consistent with those obtained by previous authors who have shown that the relative abundance of glucose increased during a daytime emersion period [17]. Overall, it was considered that the majority of the glucosyl units were components of the storage polymers chrysolaminaran, composed of (1→ 3) β -glucan chains [40]. Concerning the other sugars

detected and their levels, the results were fairly close to those already observed in the literature.

Conclusion

The aim of this work was to study the dynamics and biochemical compositions of EPS, produced on an intertidal mudflat, during the development of a microphytobenthic biofilm. This study highlighted the complexity involved in collecting and separating the different types of present EPS. The extraction procedure allowed focusing more accurately on the bound fractions, composed of specific carbohydrate monomers, *i.e.* large amounts of deoxy sugars and uronic acids. The changes in bound ECPS composition, especially through the levels of rhamnose, mannose or galacturonic acid, could play a major role during the development of the microphytobenthic biofilm by increasing binding forces and cell-cell interactions. The lack of fucose was a surprising observation and was correlated to the sampling period (winter) and the physiological state of the growing biofilm. Besides, the presence of inositol, detected for a first time *in situ*, was quite interesting. This sugar is commonly used for GC/MS analysis as an internal standard (*myo*-inositol) and this could explain why it was not identified as a component of ECPS. Significant amounts of this growing factor for bacteria were measured in the colloidal fractions, a fraction known as being a direct nutrient source for the heterotrophic bacteria constituting the microphytobenthic biofilm. In this way, the results showed that the colloidal fractions were not only used as a C-source but that they could have an impact on ion sequestration owing to high level of uronic acids. Finally, this study only focused on one sampling campaign during winter. It was shown that the greatest variations in sediment dynamics resulted from seasonal changes, modifying the concentration, type and structure of EPS accordingly (Underwood and Paterson, 2003). So, it should be interesting to check if these variations could be observed on this intertidal bay during summer.

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Table 1. Composition ($\mu\text{g}/\mu\text{g chl } a$) in uronic acids, sulfates and proteins of the different fractions extracted by the Dowex method from the Marennes-Oléron mudflat.

Fractions (LMW + HMW)	Colloidal EPS			Bound EPS			Residual polymers		
Content ($\mu\text{g}/\mu\text{g chl } a$)	Uronic acids	Sulfates	Proteins	Uronic acids	Sulfates	Proteins	Uronic acids	Sulfates	Proteins
2/18/08 - 1h	1.6 ± 0.9	0	0	1.3 ± 0.3	0	0	20 ± 5	8 ± 3	15 ± 1
2/18/08 - 2h	1.8 ± 0.3	0	0	1.4 ± 0.3	0	0	19 ± 7	8.0 ± 6	26 ± 6
2/18/08 - 3h	1.6 ± 0.9	0	0	1.4 ± 0.6	0	0	21.0 ± 6	9 ± 0.4	16 ± 0.7
2/18/08 - 4h	-	-	-	-	-	-	-	-	-
2/19/08 - 1h	1.2 ± 0.6	0	0	0.7 ± 0.3	0	0	28 ± 6	7 ± 2.0	21 ± 13
2/19/08 - 2h	1.1 ± 0.3	0	0	1.4 ± 0.8	0	0	19 ± 7	5 ± 3	18 ± 4
2/19/08 - 3h	1.5 ± 0.5	0	0	1.5 ± 0.6	0	0	25 ± 7	8 ± 1.0	20 ± 4
2/19/08 - 4h	-	-	-	-	-	-	-	-	-
2/20/08 - 1h	1.4 ± 0.5	0	0	1.3 ± 0.3	0	0	23 ± 9	9 ± 5	19 ± 4
2/20/08 - 2h	1.2 ± 0.3	0	0	0.9 ± 0.4	0	0	21 ± 15	11 ± 0.7	19 ± 8
2/20/08 - 3h	1.5 ± 0.4	0	0	0.8 ± 0.2	0	0	21 ± 9	10 ± 5	20 ± 11
2/20/08 - 4h	1.5 ± 0.3	0	0	0.9 ± 0.2	0	0	17 ± 5	12 ± 7	24 ± 8

$\pm$: deviations were calculated from the heterogeneity of the different sampling squares and not from the true replicates of the biochemical analyses (<5%)

586 Figure 1. Station where samples of surficial intertidal sediment were collected: Brouage
587 mudflat.

588 Figure 2. Light climate (A) measured each minute throughout the sampling, cell density
589 associated with Chl *a* in the first 10mm (B) and Chl *a* in the first 2mm (C) of the sediment.

590 Figure 3. Carbohydrate concentration ($\mu\text{g glc equivs.}\mu\text{g chl } a^{-1}$) for colloidal (A), bound (B)
591 and residual (C) fractions depending on the emerged period.

592 Figure 4. Distribution of LMW (% w/w) for colloidal (A), bound (B) and residual (C)
593 fractions depending on the emerged period.

594 Figure 5. Monosaccharide composition (average for the 3 sampling sites) of the different
595 fractions extracted from the mudflat. The variability within true sample replicates was less
596 than 5%. Unknown: indicates the sum of traces which were not identified.

597 Figure 6. GC-MS chromatogram of carbohydrates detected in an HMW bound fraction.
598 Ionization: Electronic Impact (EI, 70eV). Target ion: 40-650 m/z.

599 Figure 7. EPS types and location in the Marennes-Oléron mudflat, based on Underwood and
600 Paterson's (2003) conceptual model for benthic diatom exopolymères. Composition in sugars
601 (%) was averaged over the emersion period.

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FIGURE 1

FIGURE 2

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FIGURE 3

FIGURE 4

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