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 epipelic 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 Weigh (LMW) and High Molecular Weigh (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).

Environmental parameters

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

Materials

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

EPS Extraction

120 The extraction method [37] was done immediately after sampling and sediment mixing in the
121 field. 20 mL of fresh mudflat was continuously mixed with 20 mL of Artificial Sea Water
122 (ASW 30 Practical Salinity Units) during 1 h in darkness at 4 °C and then centrifuged at 3500
123 g and 4 °C for 10 min. The supernatant (a) containing colloidal EPS was collected and stored
124 at 4 °C. 20 mL of ASW and 1 g of activated Dowex (Marathon C, activated in Phosphate
125 Buffer Saline for 1 h in the dark) was added to the sediment pellet (b). The samples were
126 mixed gently at 4 °C for 1 h in the dark and then centrifuged at 3500 g and 4 °C for 10 min. A
127 supernatant containing the bound EPS (c) and a cap containing intracellular and residual
128 polymers (d) were obtained. The cap was then frozen. The residual polymers were extracted
129 from the frozen sediment samples (d), by sonication at 100 W for 3 min on ice after
130 resuspension in 20 mL in ASW.

131 For each fraction (colloidal, bound and residual polymers), absolute ethanol at -20 °C was
132 added to the sample (a) to obtain a final ethanol concentration of 75 % (v/v). The solution was
133 gently mixed and stored overnight at -20 °C. The solution was then centrifuged at 3500 g and
134 4 °C for 15 min to obtain a supernatant (Low Molecular Weight, LMW fraction) and a
135 precipitate pellet (High Molecular Weight, HMW fraction). Finally, the fractions were dried
136 under air flow and stored at -20 °C.

137 *Biochemical analysis of EPS and carbohydrate fractions*

138 Total sugar content was determined using the phenol-sulfuric acid assay, using glucose as a
139 standard [10]. Briefly, 1 mL of 5 % phenol and 5 mL sulfuric acid were added to 1 mL EPS
140 solution, previously resuspended in ultra-pure water, and vortexed. Measurements were read
141 after 30 min with a spectrophotometer at 485 nm. Total sugar amounts for the fractions were
142 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/Cl_2 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/Cl_2 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/Cl_2 – 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

166 Prior to carbohydrate characterization by GC/MS, EPS fractions were solubilized in 5 mL of
167 ultrapure water, dialyzed (6-8 KDa) and freeze-dried [2]. EPS were then dissolved in 2 M
168 HCl at 50 mg/mL and heated at 90 °C for 4 h. The preparation (which contained mostly ECPS
169 monomers) was then freeze-dried and stored at -20 °C. Analysis of the carbohydrate fractions
170 were carried out by GC/MS using a Varian CP-3800 GC/Varian Saturn 2000. 400 µL of
171 pyridine and 400 µL of BSTFA: TMCS (99: 1) was added to 2 mg of purified
172 monosaccharides. The solution was mixed for 2 h at room temperature, then injected into a
173 DB-1701 J&W Scientific column (30 m, 0.32 mm, 1 µm) at a flow of 1 mL/min. The helium
174 pressure was 8.8 psi. The temperature of the injector was set at 250 °C. The rise in
175 temperature in the oven was programmed for a first step at 150 °C for 0 min, then an
176 increment of 10 °C/min up to 200 °C with a final step at 200 °C for 35 min. The ionization
177 was performed by Electronic Impact (EI, 70 eV), the trap temperature was set at 150 °C and
178 the target ion was fixed at 40-650 m/z [29]. Each monosaccharide amount measured by
179 GC/MS was expressed depending on the total carbohydrate amounts measured by
180 biochemical assays, while considering the sampling date and the emersion time.

181 *Statistical analysis*

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

Results

Environmental parameters

Light measurements showed the higher brightness during the first day of sampling (1000 µmol photons . m^{-2} . s^{-1}) (Fig. 2A). The most important brightness peaks were obtained for the other days around 12-14h (600 to 800 µmol photons . m^{-2} . 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 µg/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 µg sugar . µg chl a^{-1} (Fig. 3A). The results showed that the amounts of LMW total sugars remained constant around 3.5 µg sugar . µg chl a^{-1} during the sampling campaign (except for 2/20/08-4h, with a concentration close to 5.5 µg sugar . µg chl a^{-1}). HMW total sugar quantities were slightly lower (3 µg sugar . µg chl a^{-1} on average, with an increase for 2/20/08-4h to 5 µg sugar . µg 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

215 respectively) (Fig. 3B). Besides, no significant effect of the spatial heterogeneity was found
216 for LMW and HMW bound carbohydrate concentrations (p values [5 %] = 0.957 and 0.324
217 respectively). The LMW total sugar quantities in bound fractions tended to increase during
218 the emerged phase. This trend was especially pronounced for the period 2/20/08 1 h - 4 h
219 since the quantity nearly doubled (6 µg to 13 µg sugar . µg chl a^{-1}) (p value for day 2/20/08 [5
220 %] = 0.028). Concerning the residual fractions (Fig. 3C), there was a good level of
221 repeatability for the LMW carbohydrate amounts between the sampling squares since no
222 significant difference was found (p value [5 %] = 0.556). It is difficult to highlight a particular
223 trend, except a stagnation of LMW carbohydrate amounts (around 20µg sugar . µg chl a^{-1}).
224 The amounts of HMW carbohydrate quantities in the residual fractions were found to increase
225 but not significantly during the emersion of the sediment (p values for days 2/18-19-20/08 [5
226 %] = 0.576, 0.976 and 0.057 respectively), at higher concentrations (30 to 75 µg sugar . µg
227 chl a^{-1}).

228 The change in distribution of total carbohydrates between LMW and HMW for the colloidal,
229 bound and residual fractions is presented in Fig. 4. The LMW colloidal carbohydrates were
230 slightly and significantly more abundant than the HMW colloidal carbohydrates (p value [5
231 %] = 0.003). For the bound fractions, the carbohydrates were produced in very precise
232 quantities as indicated by the low standard deviation between the different sampling sites. The
233 bound carbohydrates seem to be produced at a constant and significant ratio of 90/10:
234 LMW/HMW (p value [5 %] < 0.0001), compared with 55/45 for the colloidal fractions. The
235 residual fractions were essentially composed of HMW compounds, *i.e.* 60-70 % for total
236 carbohydrates.

237 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 µg . µg 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

263 HMW fractions, rhamnose represented about 35 % of the total carbohydrates. The percentage
264 of xylose was similar in LMW and HMW bound fractions (± 25 %). The percentage of
265 galacturonic acid remained constant in HMW fractions at around 16 % during the emersion.
266 The monosaccharide composition of residual fractions was different compared to colloidal
267 and bound fractions (Fig. 5C). The concentration of each monosaccharide varied widely
268 during the emerged periods, making it difficult to define a typical composition. For the LMW
269 fractions, the amount of rhamnose was relatively constant during the first hours of emersion
270 (± 10 %) and decreased drastically to 1 % during the last hour. A similar observation can be
271 made for the percentage of xylose in these fractions (10 % to 0.7 %). In addition, the
272 percentage of glucose followed an amazing trend during the emerged periods, decreasing
273 slightly during the first three hours of emersion (18 % to 12 %) and dramatically increasing
274 during the last hour to a level close to 43 % or almost half of the total monosaccharides.
275 Similar observations were made for the HMW fractions, particularly regarding the changing
276 levels of rhamnose (11 to 5 %) and glucose (31 to 13 % during the first three hours then an
277 increase to 59 % during the fourth hour). Finally, the increase in the levels of unknown peaks
278 suggests that the LMW and HMW residual fractions are really complex.

279 ECPS dynamics and bacteria abundance

280 The sediment was more rich in bacteria during the day 2/19/08 (around $5e^{+9}$ cells . g^{-1} dry
281 sediment) (Fig. 2B). The day 2/18/08 showed the lowest concentrations of bacteria ($1e^{+9}$ cells
282 . g^{-1} dry sediment). A decrease in the amount of bacteria was observed during the 2/20/08
283 ($5e^{+9}$ to $2.5e^{+9}$ cells . g^{-1} dry sediment), with significant differences found for the bacterial
284 densities at 0h, 1h and 3h of emersion (P value [5 %] = 0.013). During this period, a strong
285 increase in bound LMW carbohydrates in the sediment was observed (Fig. 2B). Nevertheless,
286 no significant correlation was found between bacteria and bound LMW carbohydrates (r =-

287 0.21, R^2 = 0.044), bound HMW carbohydrates (r =0.186, R^2 = 0.035) and more generally
288 bound (LMW + HMW) carbohydrates (r = -0.17, R^2 = 0.029).

289 **Discussion**

290 Methodological limitation

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

301 ECPS dynamics during the biofilm development process

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

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

310 biofilm matrix owing to their physico-chemical properties [1, 4]. Colloidal carbohydrates
311 were consistently produced during the three days of analysis (Fig. 3A). No particular trend
312 was found during the emerged periods, in contrast to the results obtained by Taylor et al.
313 (1999) which showed an increase in colloidal carbohydrate levels towards the end of the
314 emerged periods. It is noteworthy that Taylor worked on EPS fractions containing colloidal
315 EPS and internal polymers. LMW bound carbohydrate amounts strongly increased towards
316 the end of the emerged period (Fig. 3B) in contrast to HMW bound carbohydrates. Many
317 authors showed an increase in bound carbohydrate amounts during the tidal exposure period
318 [17, 32, 38]. Bound fractions were composed of 90 % LMW carbohydrates and this
319 percentage was extremely constant throughout the sampling period (Fig. 5B). This high ratio
320 of LMW carbohydrates could be attributed to the acidic properties of the Dowex which could
321 hydrolyze a part of HMW ECPS, thus producing an overestimation of LMW carbohydrates.
322 Cell-cell and cell-substratum interactions are facilitated by the production of tightly bound
323 carbohydrates [4, 42] through the presence of uronic acids (Table 1). The anionic properties
324 of bound ECPS, conferred by uronic acids, could allow association of divalent cations,
325 providing greater binding forces during the biofilm development process [36].

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

334 Changes in ECPS composition and ecological relevance

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

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

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

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

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

381 Besides, the presence of inositol (8 – 15 %) was also interesting, given that no author has
382 highlighted it. This molecule plays different and important roles as structural basis for a
383 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”.

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

413

414 Residual fractions and C-storage

415 LMW and HMW residual fractions (Fig. 7) could correspond to intracellular and cell wall
416 associated polymers but also consist of a mixture of different refractory EPS not totally
417 extracted by the Dowex-method. Fractions could be compared to a part of HW and all HB
418 fractions of Abdullahi et al. (2006) or Hanlon et al. (2006). The carbohydrate content was
419 composed of glucose (18 to 43 %), galacturonic acid (19 %), mannose (18 %), xylose (10 %)
420 and rhamnose (10 %). The observations of many authors indicated that, these fractions were
421 very complex, due to the presence of proteins and sulfated groups, and the different
422 monosaccharides detected could be involved in the formation of structural polymers,
423 glycolipids or proteoglycans [24]. The important point to highlight was the slight decrease in
424 the percentage of glucose during the first hours of emersion. However, towards the end of the
425 emerged periods, the quantity of glucose doubled or tripled. This observation could be
426 correlated to the capacity of diatoms “to sense the end of emersion”, by storing a maximum
427 amount of carbon for their metabolism to survive during periods of darkness. This result was
428 partly consistent with those obtained by previous authors who have shown that the relative
429 abundance of glucose increased during a daytime emersion period [17]. Overall, it was
430 considered that the majority of the glucosyl units were components of the storage polymers
431 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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577 formation of transparent exopolymer particles by bubble adsorption of seawater. Limnol
578 Oceanogr 43:1860-1871

579

580 Table 1. Composition (µg/µg chl*a*) in uronic acids, sulfates and proteins of the different
581 fractions extracted by the Dowex method from the Marennes-Oléron mudflat.

582

Fractions (LMW + HMW)	**Colloidal EPS**			**Bound EPS**			**Residual polymers**		
Content (µg/µg 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

583 *±: deviations were calculated from the heterogeneity of the different sampling squares and not from the true*
584 *replicates of the biochemical analyses (<5%)*

585

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 (µg glc equivs.µg 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.

602

603

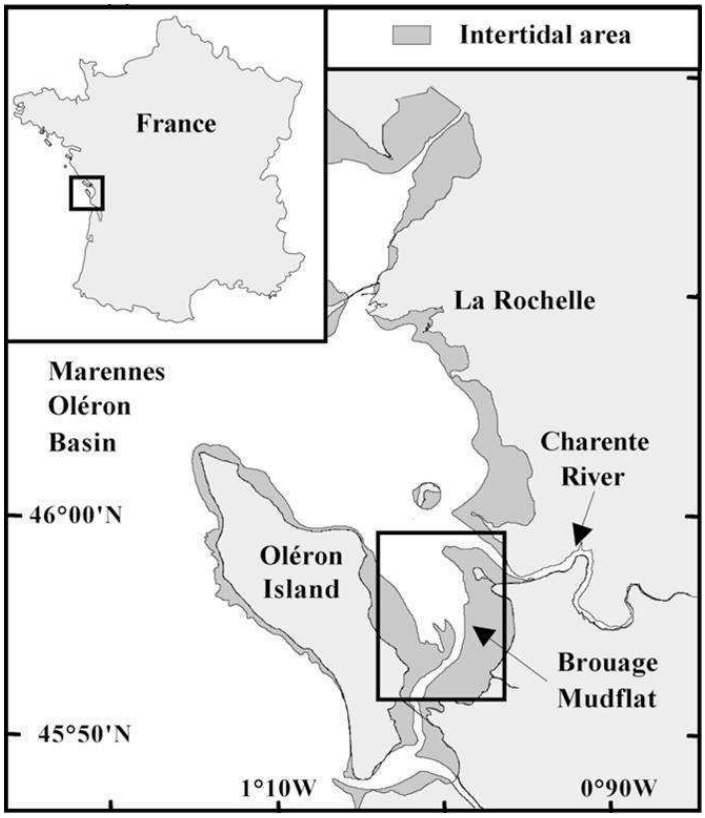
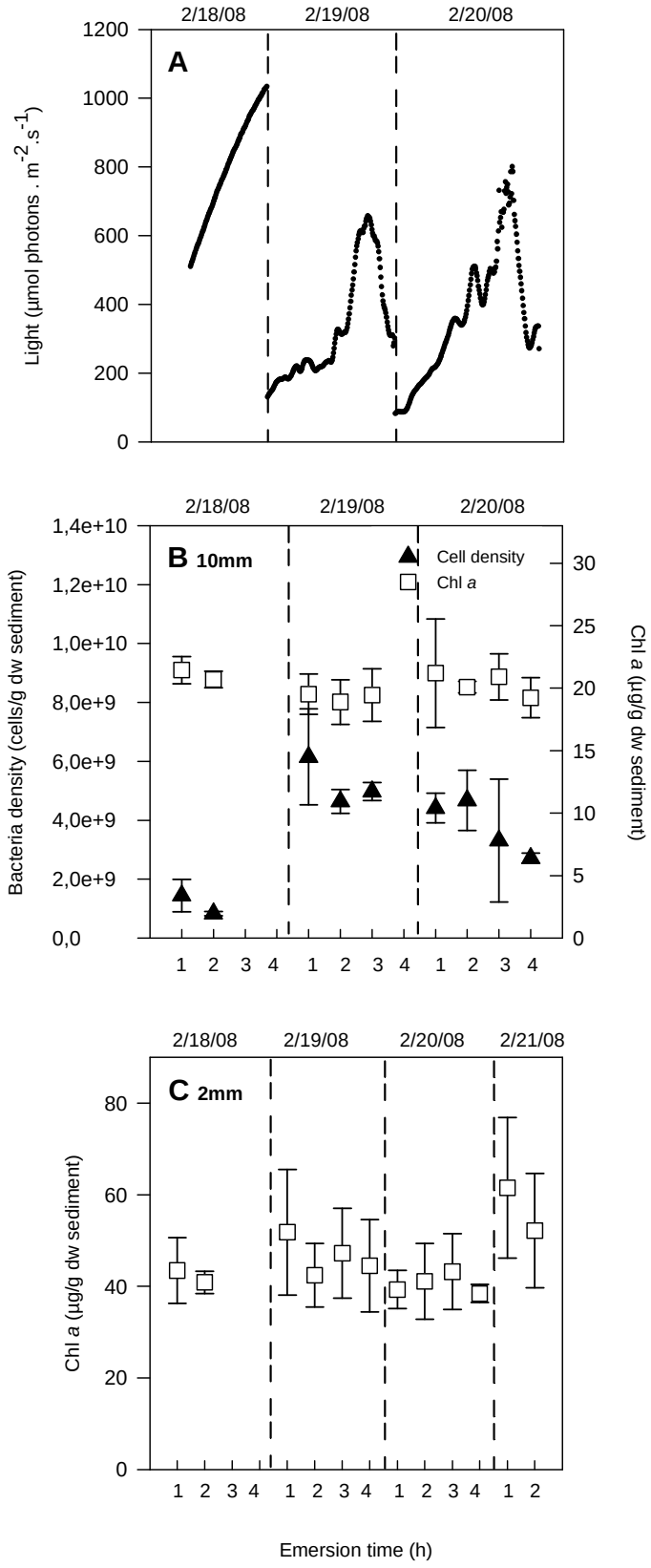

Intertidal area
France
La Rochelle
Marennes
Oléron
Basin
Charente
River
46°00'N
Oléron
Island
Brouage
Mudflat
45°50'N
1°10W
0°90W

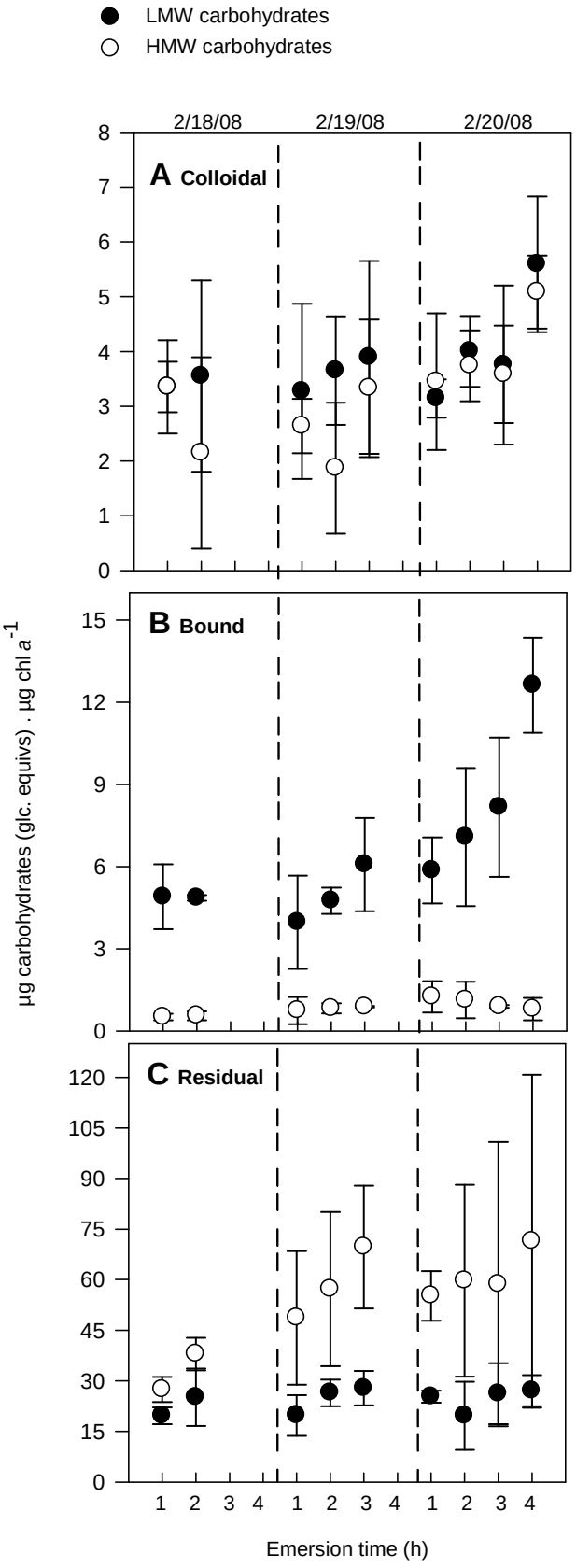
FIGURE 1



608

609 FIGURE 2

610



611

612 FIGURE 3

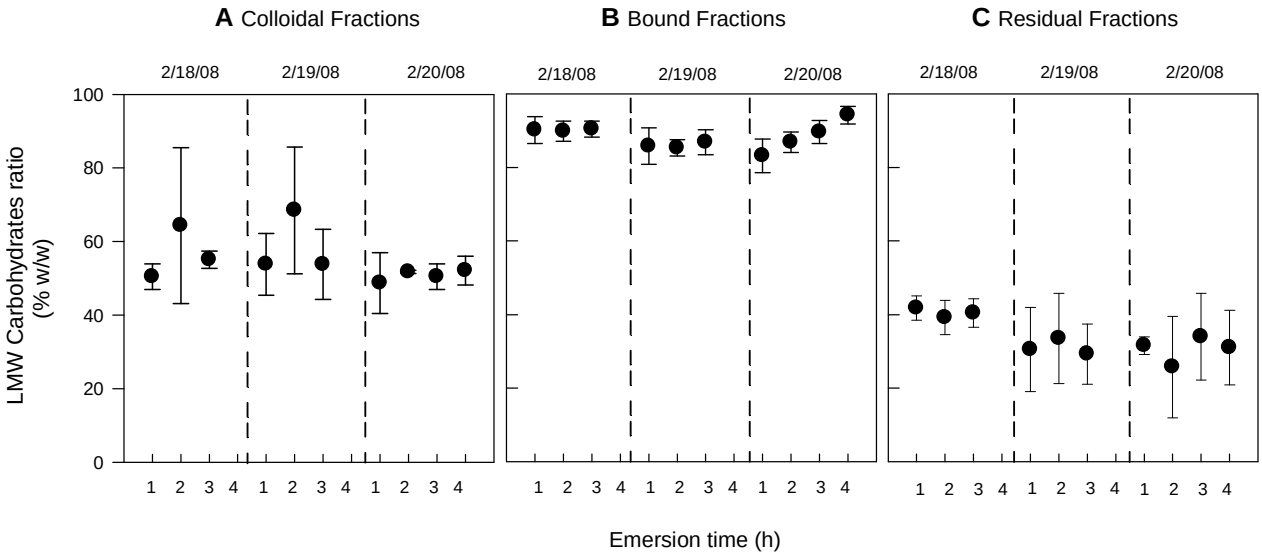
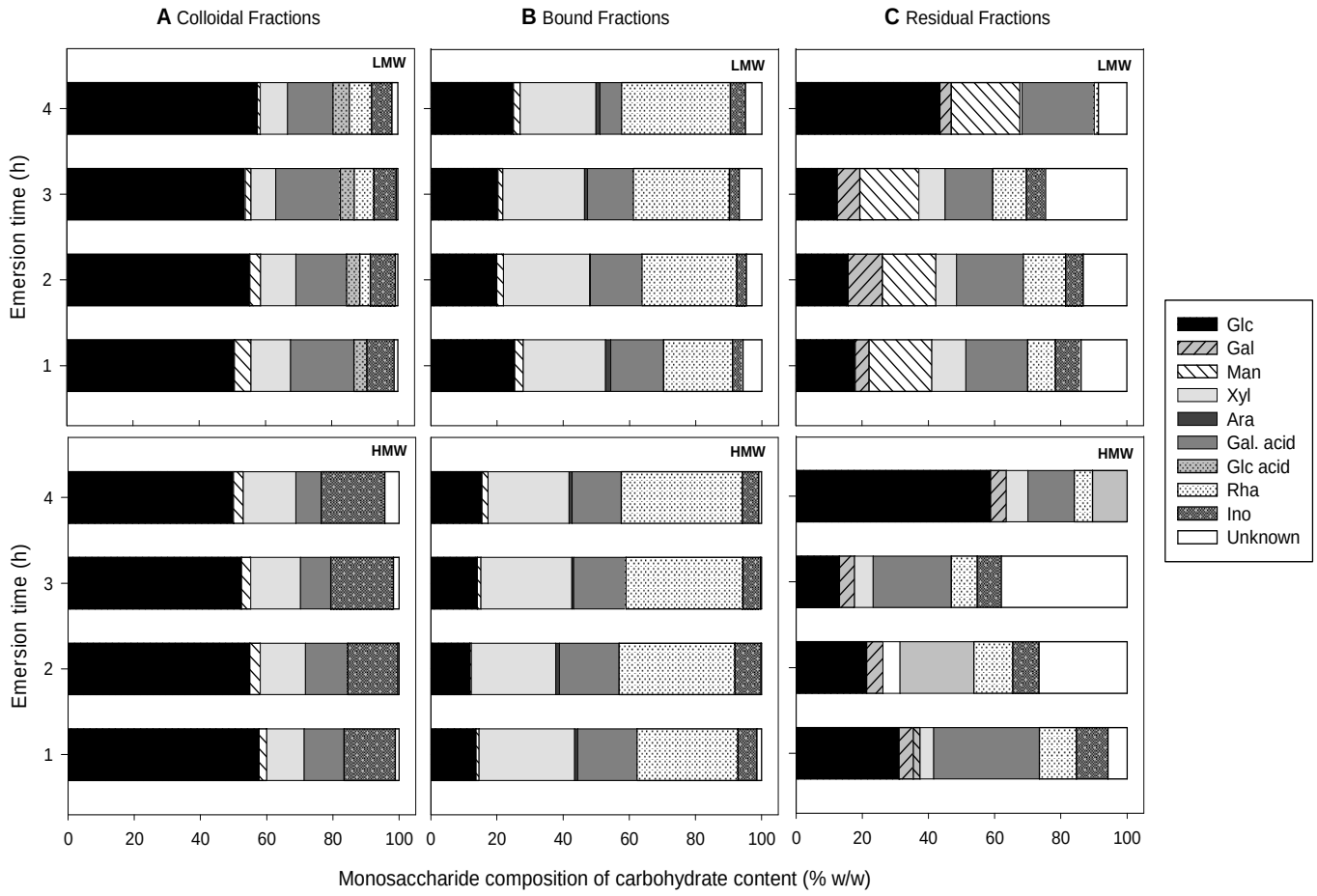

A Colloidal Fractions
B Bound Fractions
C Residual Fractions
2/18/08
2/19/08
2/20/08
LMW Carbohydrates ratio (% w/w)
0
20
40
60
80
100
1 2 3 4
Emersion time (h)

FIGURE 4

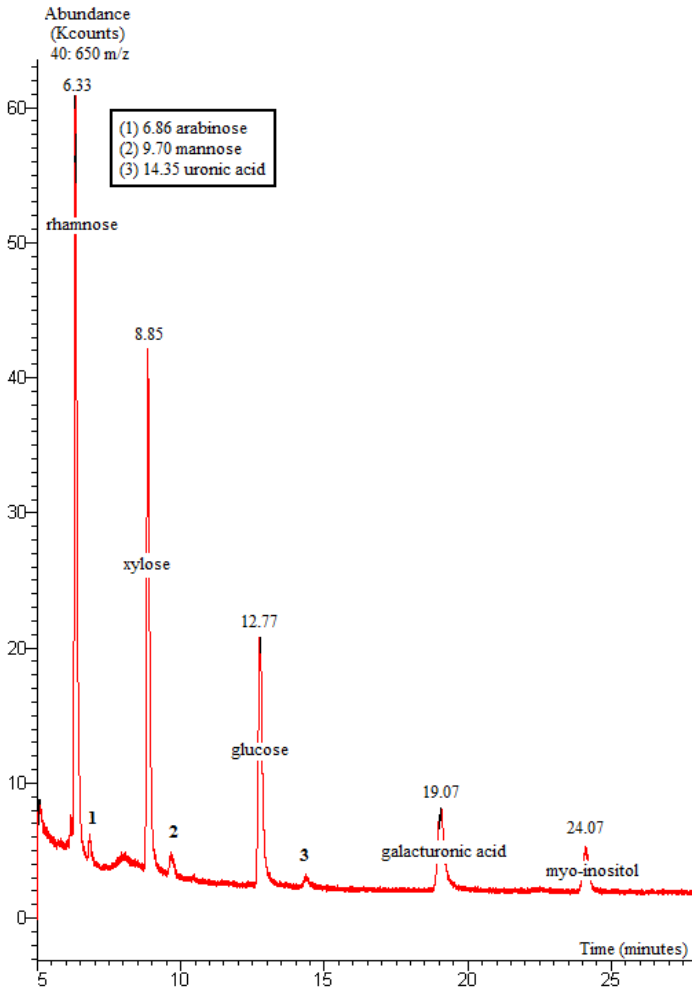
617



618

619 FIGURE 5

620

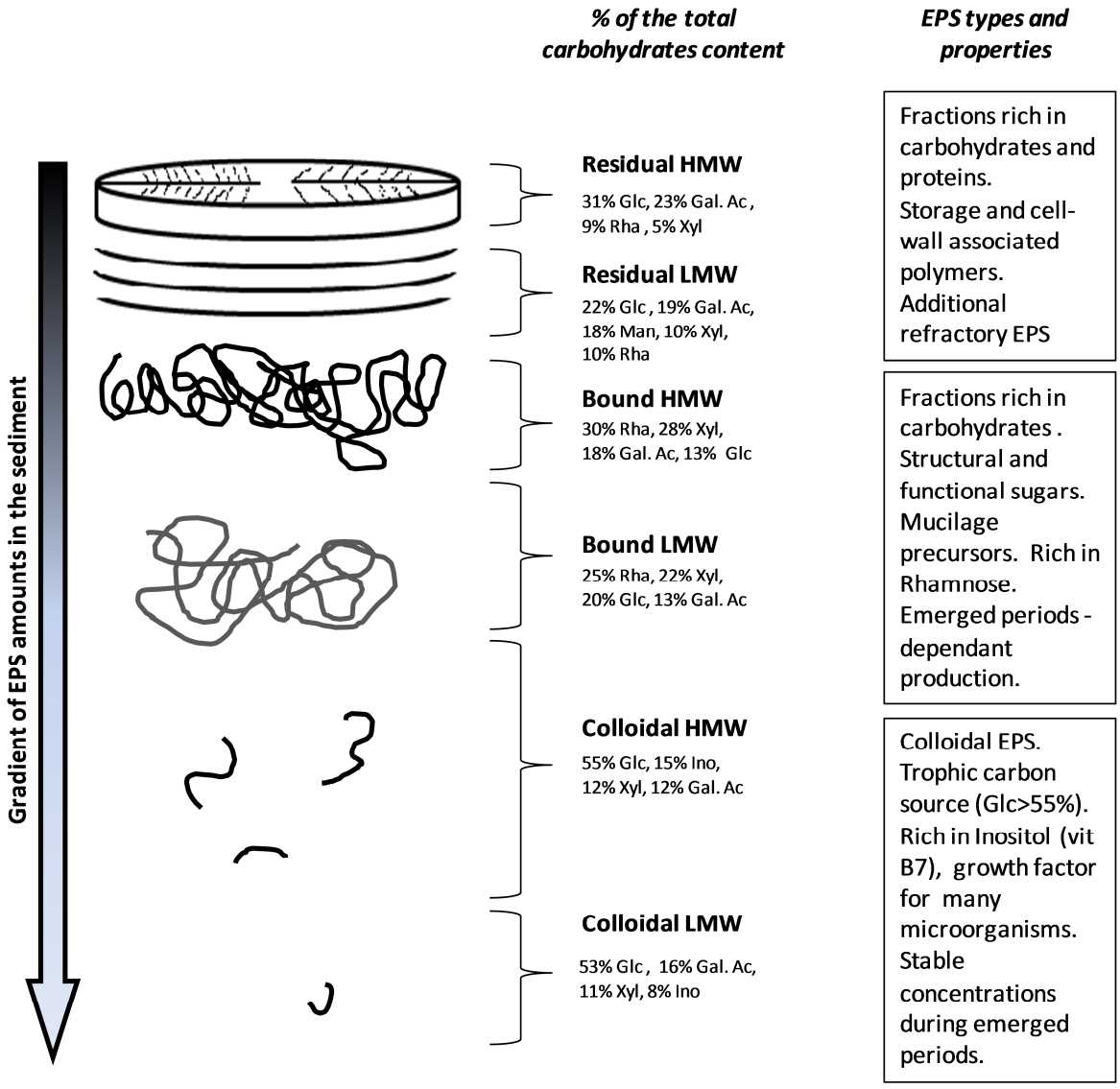


621

622

623 FIGURE 6

624



625

626

627 FIGURE 7

628

629