



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

Guillaume Pierre, Jean-Michel Zhao, Francis Orvain, Christine Dupuy, Géraldine Klein, Marianne Graber, Thierry Maugard

► To cite this version:

Guillaume Pierre, Jean-Michel Zhao, Francis Orvain, Christine Dupuy, Géraldine Klein, et al.. Seasonal dynamics of extracellular polymeric substances (EPS) in surface sediments of a diatom-dominated intertidal mudflat (Marennes-Oléron, France).. Journal of Sea Research (JSR), 2013, 92, pp.26-35. 10.1016/j.seares.2013.07.018 . hal-01070408

HAL Id: hal-01070408

<https://hal.science/hal-01070408>

Submitted on 7 Oct 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Guillaume Pierre ^{1,2*}, **Jean-Michel Zhao** ², **Francis Orvain** ³, **Christine Dupuy** ²,
Marianne Graber ², **Thierry Maugard** ²

(1) Clermont Université, Université Blaise Pascal, Institut Pascal, UMR 6602 CNRS - Polytech Clermont-Ferrand, 24 avenue des Landais, BP206, F-63174 Aubière cedex, France

(2) UMR 7266 CNRS - ULR LIENSs. Université de La Rochelle, UFR Sciences, Bâtiment Marie Curie, avenue Michel Crépeau, 17042 La Rochelle, France.

(3) Université de Caen Basse-Normandie, CNRS FRE 3484 BioMEA, esplanade de la Paix, 14032 Caen, France.

* Tel: +33 (0) 4 73 40 74 22 Fax: +33 (0) 4 73 40 78 29 E-mail: guillaume.pierre@univ-bpclermont.fr

Abstract

Numerous field-based investigations have highlighted that the production of Extracellular Polymeric Substances (EPS) is physico-chemically and ecologically essential 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, emersion time, luminosity, C:N ratio or 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 was more important than during summer. Yet, more proteins were found during summer especially for the colloidal and bound fractions. 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, as salinity, pore water, tidal coefficient could have a significant impact on the concentration rates and pathways of carbohydrates.

Key words: Extracellular polymeric substances (EPS), carbohydrates, diatoms, seasonal impacts, mudflat

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 (Falcatore 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) composing benthic biofilms. 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., 1994). 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 microenvironment of the benthic biofilms (Underwood et al., 1995) and are used by different microorganisms, *e. g.* the glucose-rich exudates consumed by heterotrophic bacteria (van Duyl et al., 1999; Hofmann et al. 2009). 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 epipelagic diatoms (Stal and Défarge, 2005), in cells/cells or cells/substratum adhesion (Wimpenny et al. 2000) and sediment biostabilisation (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 ; Pierre et al., 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 (Decho, 2000; Bellinger et al., 2005). 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 investigate the temporal distribution and compositional changes of EPS, in relation to seasonal dynamics. A challenging aim was also to compile numerous data obtained on the same field to clearly show the impact of seasons on the EPS composition of a microphytobenthic biofilm and, *a fortiori*, on the trophic involvement of intertidal mudflats. 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 microphytobenthic 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 (2m-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 1cm 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 chllophyll *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, C:N ratio were also followed. Enumeration of bacteria was performed by microscopy, after 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) labeling (x 1000, Axioskop, Zeiss) using the method of Porter and Feig (1980). All missing data were due to experiment constraints.

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, 1 μ m) for Gas Chromatography-Mass Spectrometry analysis (GC/MS) was obtained from Agilent.

2.4. EPS extraction and fractionation

The 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) during 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., 1985).

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&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.

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), the term “carbohydrates” refers to the total sugars (neutral and acidic sugars) determined by Dubois assays and GC/MS. The term “uronic acids” corresponds only to the uronic assays clearly measured by the BCA method or GC/MS analyses. Concerning the abbreviations, W and S correspond to winter and summer, the two sampling periods. LMW and HMW refer to the size of the carbohydrates: low molecular weight and high molecular weight. CC, BC and RC refer to the carbohydrates

extracted from the three main fractions: colloidal carbohydrates (CC) are water-extractable, bound carbohydrates (BC) are obtained by using Dowex Marathon C, and residual carbohydrates (RC) correspond to the fractions rich in intracellular carbohydrates and refractory EPS.

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 (Fig. 3). At this step, it is noteworthy that the sugar amounts were normalized to Chl *a* in order to overestimate diatom EPS production, compared to other EPS sources (Haubois et al., 2005). In average and regardless the emersion time, more colloidal carbohydrates were extracted from the sediment in winter ($5.28 \mu\text{g} \cdot \mu\text{g chl } a^{-1}$) than summer ($2.04 \mu\text{g} \cdot \mu\text{g chl } a^{-1}$). The same observations were done for the bound and residual carbohydrates since 2.5 times more carbohydrates were found in the winter samples (Fig. 3A). 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 epipellic diatoms, which move up to the sediment (Serôdio et al., 1997). Microphytobenthic
 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 $1600 \pm 400 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ in
 summer and $440 \pm 250 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ in winter. The level of chl *a* measured on the
 sediment was significantly lower ($p < 0.01$) in summer ($8.1 \pm 1.2 \mu\text{g.g dry sediment}^{-1}$) than
 in winter ($20.2 \pm 0.9 \mu\text{g.g dry sediment}^{-1}$). This difference was attributed to the intense level
 of grazing by *Peringia ulvae* whose the density was higher in summer ($17.2 \text{ ind.m}^{-2} \pm 7.08$)
 than in winter ($5.77 \text{ ind.m}^{-2} \pm 2.99$). Moreover, the classical normalization with chl *a*
 enhanced the gap between the values in summer and winter. Besides, intertidal mudflats are
 living areas whose the primary trophic production is non-negligible. Indeed, diatoms and their
 EPS production are responsible for 40 to 50 % of the total global primary production in
 marine systems (Medlin, 2002). 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 (Fig.3, B). Yet, proteins were also found in colloidal and bound fractions collected
 in summer. During summer, pore water 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 1, p
 < 0.05). Some authors have suggested that EPS (carbohydrates and proteins) could protect

microphytobenthos 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 1). These fractions could be correlated to osmoregulatory and hydration 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, in order to hour, which occur at the surface sediments during periodic tidal exposures but the patterns varied even if colloidal carbohydrates are often involved (Taylor and Paterson, 1998; Christie et al., 2000, Hofmann et al. 2009, Pierre et al. 2012). 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. 4). 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, 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. In this way, previous studies did not specifically work on colloidal EPS but on fractions containing a part of bound and residual polymers. 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 levels and production 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 (van Duyl et al., 1999; Hanlon et al. 2006; Pierre et al. 2012).

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 the amounts dropped and bound carbohydrates whose the concentrations increased during the emerged period. A 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 microphytobenthic biofilm. The role of microphytobenthos as sediment stabilizers was well documented (Underwood and Paterson, 1993; Paterson and Black, 1999, Thornton et al., 2002; Widdows and Brinsley, 2002; Spears et al., 2008) 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; Underwood and Paterson, 2003; Hofmann et al. 2009; Pierre et al., 2011). Anyway, we noted that no light-dependant production was found for the bound fractions on the contrary to colloidal fractions whose the production was tidal coefficient-dependant (Table 1, $p < 0.05$).

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, as chl *a*, light, salinity, pore water, bacterial abundance, tidal coefficient, C:N ratio, in view of understanding their involvement in EPS dynamic productions. We previously showed that salinity, pore water, light and chl *a* have to be considered when the seasonal dynamics of EPS are studied. But it was also interesting to note that tidal coefficients could have a significant impact (Table 1) 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/microalgae, 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. 5). 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 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, as nematods, hydrobia or herbivorous zooplankton, which are more abundant and more active in summer. Thus, colloidal carbohydrates dropped during the emersion time (Fig. 4) but glucose content were higher (Fig. 5). It is therefore possible that carbohydrate EPS are selectively used by heterotrophic bacteria depending on season. In this way, bacteria could use the different monosaccharide as substrate but with different degrees of induction. Besides, the ratios of structural hexoses (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., 2011) and other studies (Hanlon et al., 2006). Owing to their surface-active properties, deoxy sugars can promote the biostabilisation of sediments (Zhou et al, 1998; Girollo et al, 2003) by facilitating the coagulation of particles and macromolecules (Khodse et al., 2007). Rhamnose could also play a role of biochemical sensor in microphytobenthic 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 biostabilisation of the sediment in summer (LMW BC: 25.6 %, HMW BC: 18 %).

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

319 and bound fractions did not contain fucose, in contrary to many works (Abdullahi et al., 2006;
320 Hanlon et al., 2006; Hofmann et al., 2009; Takahashi et al., 2009). It is relevant to highlight
321 that fucose is a monosaccharide involved in the metabolic pathways occurring during the
322 degradation of β -1,3-glucan storage in diatom cells (Granum, 2002), which could explain its
323 unique presence in residual fractions.

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

330 A principal component analysis (ACP) along dominant sugar vectors was performed to
331 clearly identify the differences in monosaccharide distributions of the EPS fractions (Fig. 6).
332 Three distinct clusters were highlighted by ACP (C1 to C3). C1 was a cluster constituted of
333 carbohydrates rich in glucose and inositol. Colloidal carbohydrates were sorted in C1.
334 However, a significant seasonal difference was found between the monosaccharide
335 distribution of W-LMW CC and S-LMW CC. Light and pore water could play a significant
336 role in this dissimilarity (Table 1, $p < 0.05$). So, the monosaccharide distribution of LMW CC
337 could be seasonal dependant (no cluster). These fractions are extremely labile and can be
338 selectively consumed by heterotrophic bacteria. Cluster 2 represented fractions rich in
339 rhamnose, xylose and galacturonic acid. Bound fractions, excepting S-HMW BC, belonged to
340 C2. Previously, we noted that the rhamnose content was lower in S-HMW BC and we
341 suggested that this ratio could play a major impact on sediment biostabilisation.

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 (Underwood and Paterson, 2003; Hofmann et al., 2009). Moreover, this is the selective consumption of carbohydrate EPS by heterotrophic bacteria which seems to play the major role in these EPS changes (Giroldo et al., 2003). Previously, we showed that four major monosaccharides (glucose, xylose, galacturonic acid, 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 sugars 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. 7). 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 production, in term of amounts, could vary in response to environmental parameters (cf. part 3.2.). Bacterial interactions, adhesion and biofilm formation, protection against salinity or migration into the sediment could be ecological and physico-chemical phenomena involving EPS changes.

In details, the results showed that all over the emersion periods, glucose amounts stayed stable whatever 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 (Decho, 1990; Sutherland, 2001; Bhaskar et al., 2005). 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 part 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 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, 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

390 involvement in adhesion/cohesive properties and/or in the locomotive properties of diatom
391 cells which have to migrate back into the sediment at the end of emersion time. These
392 dynamic pathways were identical between winter and summer, even if the data numbers
393 allowed us to significantly confirm this hypothesis only for summer. The presence of proteins
394 during summer was correlated to an additional C-storing for diatoms cells (residual fractions)
395 and to a potential protection against high desiccation and salinity degrees. A number of
396 abiotic/biotic parameters seemed to have impact on EPS dynamics and production as salinity,
397 pore water, light or tidal coefficient. At last, GC/MS analyses showed that colloidal
398 carbohydrates were glucose-rich, bound carbohydrates were significantly composed of
399 rhamnose and residual fractions presented a wide variety of monosaccharides. All the
400 fractions were richer in glucose during summer than winter, maybe to response to greater
401 trophic needs. ACP analyses allowed refining the results and highlighted that colloidal
402 carbohydrates formed a first cluster (glc, ino). Cluster 2 (xyl, rham) was constituted of bound
403 carbohydrates and cluster 3 (fuc, gal. acid, gal, man) of residual carbohydrates. The evolution
404 of five representative monosaccharides (glc, gal. acid, xyl, rham, fuc) was also followed in
405 function of the tidal emersion periods and the results showed again that the distribution
406 pathways did not change in winter or summer in contrary to monosaccharide amounts. We
407 noted that the levels of galacturonic acid varied for colloidal and bound fractions, which could
408 have an effect on binding forces, protection (via ion sequestration for instance) and cell-cell
409 interactions in microphytobenthic biofilms. Besides, C-storage consumption and C-storing
410 (glc, fuc, man) were observed in residual fractions during the emerged periods. To conclude,
411 many results were consistent with the literature and additional information were obtained
412 concerning the influence of seasonal parameters on EPS productions and dynamics. It should
413 be interesting to check whether these conclusions could be observed on a much larger data
414 set, specifically targeting light, salinity and pore water, tidal coefficient as major parameters.

415 **Acknowledgements**

416 This study was supported by the Conseil Général of Charentes-Maritime through the Contrat
417 de Projets Etat-Région “Littoral” and the Centre National de la Recherche Scientifique. The
418 field sampling was supported by the French ANR (National Research Agency) through the
419 VASIREMI project “Trophic significance of microbial biofilms in tidal flats” (contract ANR-
420 06-BLAN-0393-01).

421 **References**

- 422 Abdullahi, A.S., Underwood, G.J.C, Gretz, M.R., 2006. Extracellular matrix assembly in
423 diatoms (Bacillariophyceae). V. Environmental effects on polysaccharide synthesis in the
424 model diatom, *Phaeodactylum tricornutum*. J. Phycol. 42, 363-378.
- 425 Andersen, T.J., 2001. Seasonal variation in erodability of two temperate microtidal mudflats.
426 Est. Coast. Shelf Sci. 49, 99-111.
- 427 Azerado, J., Henriques, M., Sillankorva, S., Oliveira, R., 2003. Extraction of exopolymers
428 from biofilm: the protective effect of glutaraldehyde. Wat. Sci. Technol. 47, 175-179.
- 429 Bellinger, B.J., Abdullahi, A.S., Gretz, M.R., Underwood, G.J.C., 2005. Biofilm polymers:
430 relationship between carbohydrate biopolymers from estuarine mudflats and unialgal cultures
431 of benthic diatoms. Aquat. Microb. Ecol. 38, 169-180.
- 432 Bhaskar, P.V., Bhosle, N.B., 2005. Microbial extracellular polymeric substances in marine
433 biogeochemical processes. Curr. Sci. 88, 45-53.
- 434 Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of
435 uronic acids. Anal. Biochem. 54, 484-489.

436 Christie, M.C., Dyer, K.R., Blanchard, G., Cramp, A., Mitchener, H.J., Paterson, D.M., 2000.
 437 Temporal and spatial distributions of moisture and organic content across a macro-tidal
 438 mudflat. *Cont. Shelf Res.* 20, 1219-1242.

439 de Brouwer, J.F.C, Stal, L.J., 2001. Short-term dynamics in microphytobenthos distribution
 440 and associated extracellular carbohydrates in surface sediments of an intertidal mudflat. *Mar.*
 441 *Ecol. Prog. Ser.* 218, 33-44.

442 de Brouwer, J.F.C, Stal, L.J., 2004. Does warm-water extraction of benthic diatoms yield
 443 extracellular polymeric substances or does it extract intracellular chrysolaminaran? *Eur. J.*
 444 *Phycol.* 39, 129-131.

445 Decho, A.W., 1990. Microbial exopolymer secretions in ocean environment: their role(s) in
 446 food webs and marine processes. *Oceanogr. Mar. Biol. Ann. Rev.* 28, 73-153.

447 Decho, A.W., 2000. Microbial biofilms intertidal systems: an overview. *Cont. Shelf Res.* 20,
 448 1257-1274.

449 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method
 450 ofr determination of sugars and related substances. *Anal. Chem.* 28, 350-356.

451 Falciatore, A., Bowler, C., 2002. Revealing the molecular secrets of marine diatoms. *Annu.*
 452 *Rev. Plant Biol.* 53, 109-130.

453 Filisetti-Cozzi, T.M., Carpita, N.C., 1991. Measurement of uronic acids without interference
 454 from neutral sugars. *Anal. Biochem.* 197, 157-162.

455 Frostick, L.E., McCave, I.N., 1979. Seasonal shifts of sediment within an estuary mediated by
 456 algal growth. *Estuar. Coast Mar. Sci.* 9, 569-576.

457 Girolardo, D., Vieira, A.A.H., Paulsen, B.S., 2003. Relative increase of deoxy sugars during
 458 microbial degradation of an extracellular polysaccharide released by a tropical freshwater
 459 *Thalassiosira* sp. (Bacillariophyceae). J. Bacteriol. 189, 7945-7947.

460 Granum, E., Mykkestad, S.M., 2001. Mobilization of β -1,3-glucan and biosynthesis of amino
 461 acids induced by NH_4^{4+} addition to N-limited cells of the marine diatom *Skeletonema costatum*
 462 (Bacillariophyceae). J. Phycol. 37, 772-782.

463 Haubois, A.G., Sylvestre, F., Guarini, J.M., Richard, P., Blanchard G., 2005. Spatio-temporal
 464 structure of the epipellic diatom assemblage from an intertidal mudflat in Marenne-Oléron
 465 Bay, France. Estuar. Coast. Shelf Sci. 64, 385-394.

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

470 Hofmann, T., Hanlon, A.R.M, Taylor, J.D., Ball, A.S., Osborn, A.M., Underwood, G.J.C.,
 471 2009. Dynamics and compositional changes in extracellular carbohydrates in estuarine
 472 sediments during degradation. Mar. Ecol. Prog. Ser. 379, 45-58.

473 Khodse, V.B., Fernandes, L., Gopalkrishna, V.V., Bhosle, N.B., Fernandes, S.G., Matondkar,
 474 S.G.P., Bhushan, R., 2007. Mar. Chem. 103, 327-346.

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

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

480 Medlin, L.K., 2002. Why silica or better yet why not silica? Speculations as to why the
 481 diatoms utilize silica as their cell wall material. *Diatom Res.* 17, 453-459.

482 Paterson, D.M., Black, K.S., 1999. Water flow, sediment dynamics and benthic biology. *Adv.*
 483 *Ecol. Res.* 29, 155-193.

484 Pierre, G., Graber, M., Orvain, F., Dupuy, C., Maugard, T., 2010. Biochemical
 485 characterization of extracellular polymeric substances extracted from an intertidal mudflat
 486 using a cation exchange resin. *Biochem. Syst. Ecol.* 38, 917-923.

487 Pierre, G., Graber, M., Rafiliposon B.A., Dupuy, C., Orvain, F., de Crignis, M., Maugard, T.,
 488 2012. Biochemical composition and changes of extracellular polysaccharides (ECPS)
 489 produced during microphytobenthic biofilm development (Marennes-Oléron, France).
 490 *Microb. Ecol.* 63, 157-169.

491 Porter, K.G., Feig, Y.S., 1980. The use of DAPI for identifying and counting aquatic
 492 microflora. *Limnol. Oceanogr.* 25, 943-948.

493 Serôdio, J., da Silva, J.M., Catarino, F., 1997. Non-destructive tracing of migratory rhythms
 494 of intertidal benthic microalgae using *in vivo* chlorophyll *a* fluorescence. *J. Phycol.* 33, 542-
 495 553.

496 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D.,
 497 Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1987. Measurement of protein using
 498 bicinchoninic acid. *Anal. Biochem.* 150, 76-85.

499 Spears, B.M., Saunders, J.E., Davidson, I., Paterson, D.M., 2008. Microalgal sediment
 500 biostabilisation along a salinity gradient in the Eden Estuary, Scotland: unraveling a paradox.
 501 *Mar. Freshwat. Res.* 59, 313-321.

502 Staats, N., de Deckere, E.M.G.T., de Winder, B., Stal, L.J., 2001. Spatial patterns of benthic
 503 diatoms, carbohydrates and mud on a tidal flat in the Ems-Dollard estuary. *Hydrobiol.* 448,
 504 107-115.

505 Stal, L.J., Défarge, C., 2005. Structure and dynamics of exopolymers in an intertidal diatom
 506 biofilm. *Geomicrob. J.* 22, 341-352.

507 Sutherland, T.F., Grant, J., Amos, C.L., 1998. The effect of carbohydrate production by the
 508 diatom *Nitzschia curvilineata* on the erodability of sediment. *Limnol. Oceanogr.* 43, 65-72.

509 Sutherland, I.W., 2001. Biofilm exopolysaccharides: a strong and sticky framework.
 510 *Microbiol.* 147, 3-9.

511 Takahashi, E., Ledauphin, J., Goux, D., Orvain, F., 2009. Optimizing extraction of
 512 extracellular polymeric substances (EPS) from benthic diatoms: comparison of the efficiency
 513 of six EPS extraction methods. *Mar. Freshwater. Res.* 60, 1201-1210.

514 Taylor, I, Paterson, D.M., 1998. Microspatial variation in carbohydrate concentrations with
 515 depth in the upper millimeters of intertidal cohesive sediments. *Est. Coast. Shelf Sci.* 46, 359-
 516 370.

517 Thornton, D.C.O., Dong, L.F., Underwood, G.J.C., Newell, D.B., 2002. Factors affecting
 518 microphytobenthic biomass, species composition and production in the Colne Estuary (UK).
 519 *Aquat. Microb. Ecol.* 27, 285-300.

520 Underwood, G.J.C, Paterson, D.M., 1993. Seasonal changes in diatom biomass, sediment
 521 stability and biogenic stabilization in the Severn estuary. *J. Mar. Biol. Ass.* 73, 871-887.

522 Underwood, G.J.C., Paterson, D.M., Parkes, R.J., 1995. The measurement of microbial
 523 carbohydrate exopolymères from intertidal sediments. *Limnol. Oceano.* 40, 1243-1253.

524 Underwood, G.J.C, Kromkamp, J., 1999. Primary production by phytoplankton and
 525 microphytobenthos in estuaries. *Adv. Ecol. Res.* 29, 93-153.

526 Underwood, G.J.C., Paterson, D.M., 2003. The importance of extracellular carbohydrate
 527 production by marine epipelagic diatoms. *Adv. Bot. Res.* 40, 184-240.

528 Underwood, G.J.C, Boulcott, M., Raines, C.A., 2004. Environmental effects on exopolymeric
 529 production by marine benthic diatoms: dynamics, changes in composition, and pathways of
 530 production. *J. Phycol.* 40, 293-304.

531 Underwood, G.J.C., Fietz, S., Papadimitriou, S., Thomas, D.N., Dieckmann, G.S., 2010.
 532 Distribution and composition of dissolved extracellular polymeric substances (EPS) in
 533 Antarctic sea ice. *Mar. Ecol. Prog. Ser.* 404, 1-19.

534 van Duyl, F.C., de Winder, B., Kop, A.J., Wollenzien, U., 1999. Tidal coupling between
 535 carbohydrate concentrations and bacterial activities in diatom-inhabited intertidal mudflats.
 536 *Mar. Ecol. Prog. Ser.* 191, 19-32.

537 Widdows, J., Brinsley, M., 2002. Impact of biotic and abiotic processes on sediment
 538 dynamics and the consequences to the structure and functioning of the intertidal zone. *J. Sea*
 539 *Res.* 48, 143-156.

540 Wimpenny, J., Manz, W., Szewzyk, U., 2000. Heterogeneity in biofilms. *FEMS Microbiol.*
 541 *Rev.* 24, 661-671.

542 Wingender, J., Neu, T.R., Flemming, H.C., 1999. What are bacterial extracellular polymeric
 543 substances?, in: Wingender, J., Neu, T.R., Stadler, P. (Eds.), *Microbial extracellular*
 544 *polymeric substances: characterization, structure and function.* Springer-Verlag, Berlin, pp. 1-
 545 15.

546 Yallop, M.L., Paterson, D.M., Wellsbury, P., 2000. Inter-relationships between rates of
547 microbial production, exopolymer production, microbial biomass and sediment stability in
548 biofilms of intertidal sediments. *Microb. Ecol.* 39, 116-127.

549 Zhou, J., Mopper, K., Passow, U., 1998. The role of surface-active carbohydrates in the
550 formation of transparent exopolymer particles by bubble adsorption of seawater. *Limnol.*
551 *Oceanogr.* 43, 1860-1871.

552

Table 1. 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). Values of r significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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

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

Figure 3. Total carbohydrate quantities ($\mu\text{g} \cdot \mu\text{g chlo } a^{-1}$) and relative contribution (% w/w) of neutral carbohydrates (light grey), uronic acid (dark grey) and proteins (grey) for each EPS fraction extracted during (A) winter and (B) summer campaigns. Bars represent the heterogeneity of the three sampling sites.

Figure 4. Comparison of carbohydrates quantities ($\mu\text{g} \cdot \text{g dry sediment}^{-1}$) 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.

Figure 5. Monosaccharide composition (% w/w) of the different fractions extracted in winter and summer. See fig 4. for abbreviations meaning.

Figure 6. Principal component analysis scatter plot grouping monosaccharide profiles of the different fractions along dominant sugar vectors. See fig 4. for abbreviations meaning.

Figure 7. 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 in fig. 6 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 4. for abbreviations meaning.

586

587 **TABLE 1**

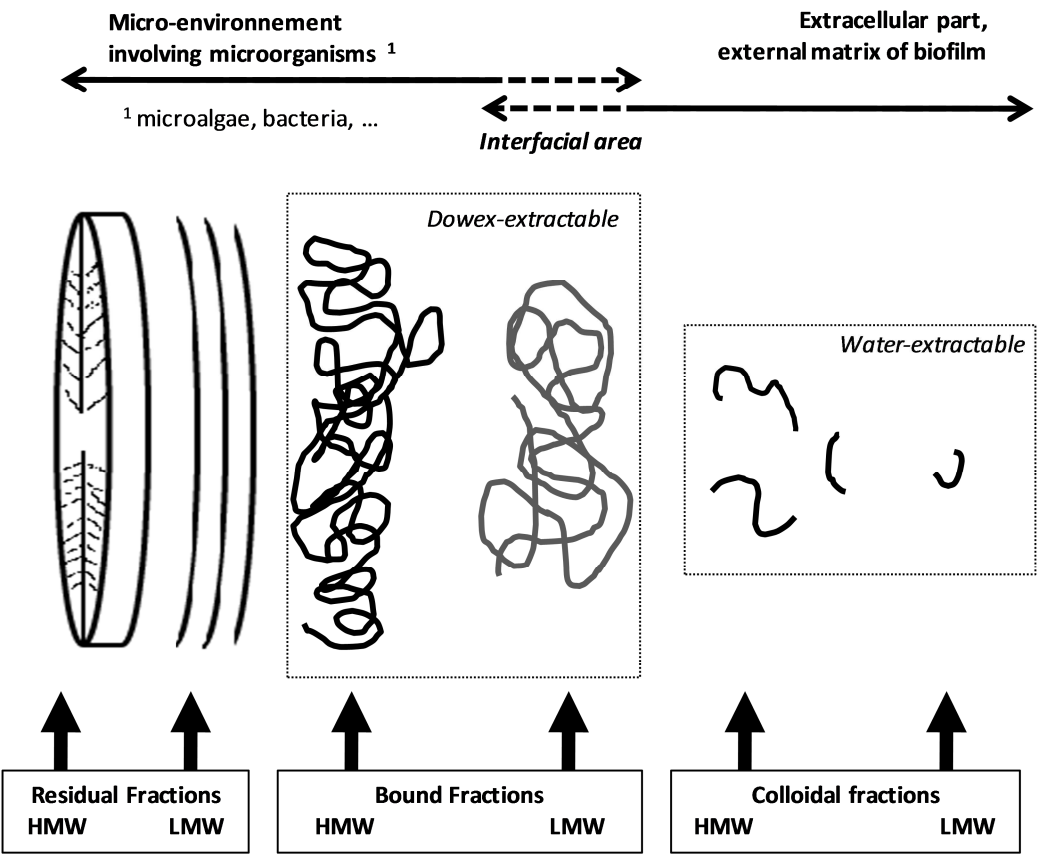
a) Winter	W-LMW CC	W-HMW CC	W-LMW BC	W-HMW BC	Light	Salinity	Pore water	Bacterial abundance	Tidal coefficient	C:N
Chl <i>a</i>	-0.114	0.291	-0.087	0.007	0.257	0.417	0.556	-0.524	-0.391	nd
W-LMW CC	x	0.656*	0.852**	-0.185	0.698*	-0.387	0.083	-0.323	0.200	nd
W-HMW CC	x	x	0.795**	0.276	0.347	-0.393	-0.176	-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 <i>a</i>	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

588 *nd: non determined*

589

590

591



592

593

594

595

FIGURE 1

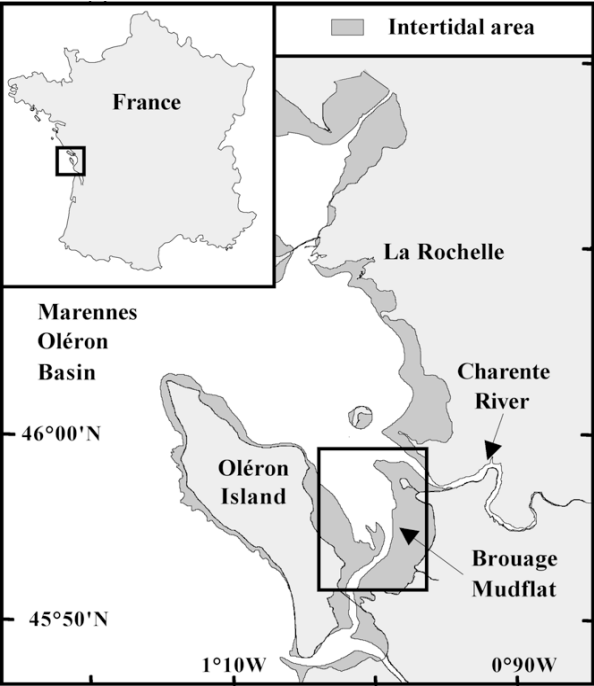


FIGURE 2

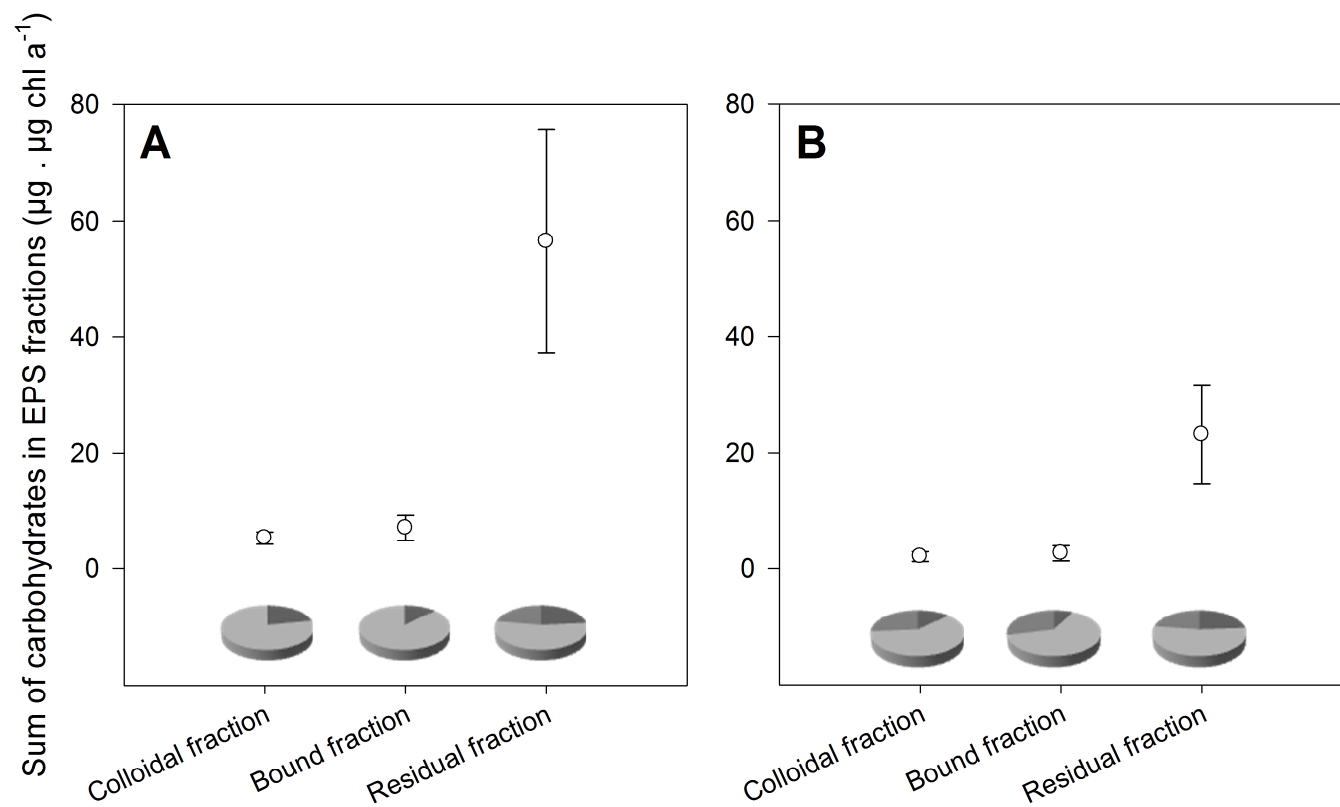


FIGURE 3

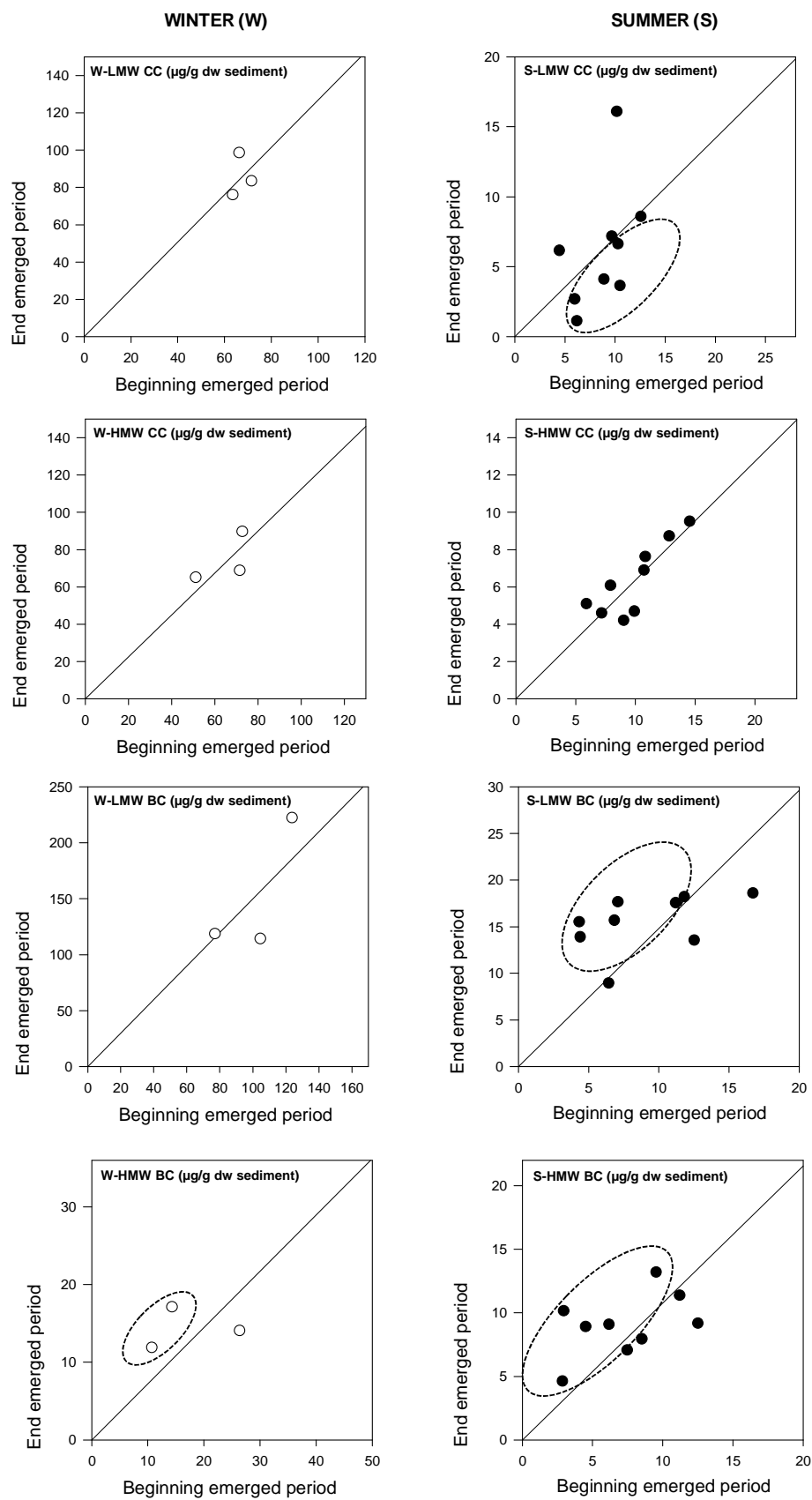
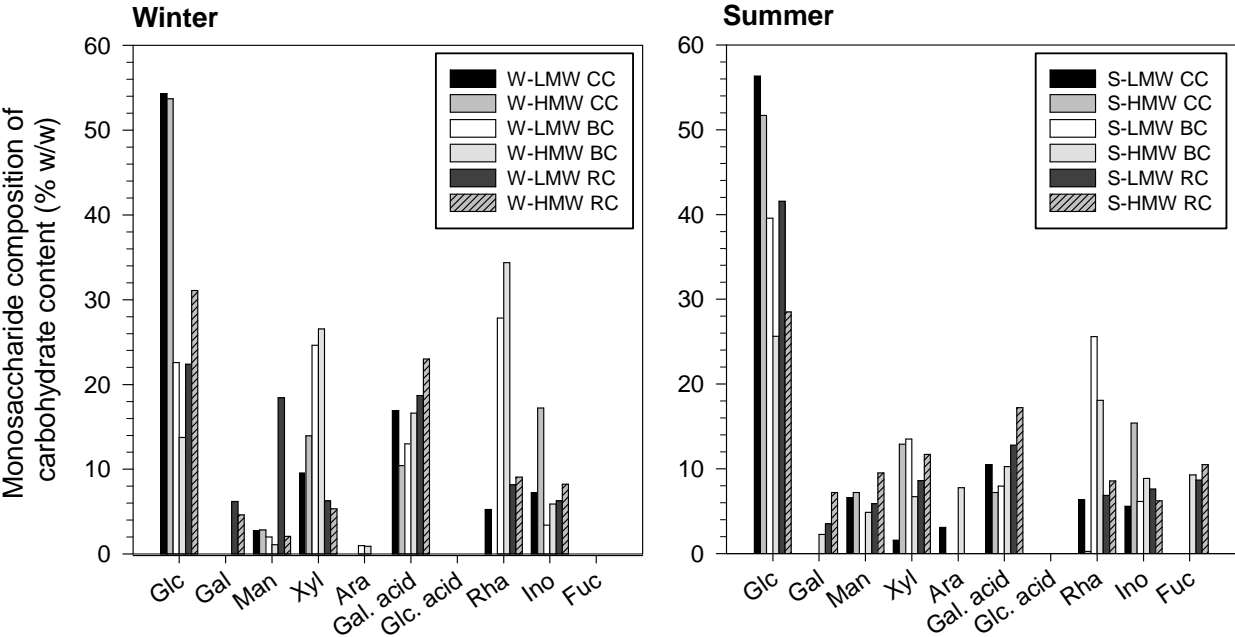


FIGURE 4

605



606

607

FIGURE 5

608

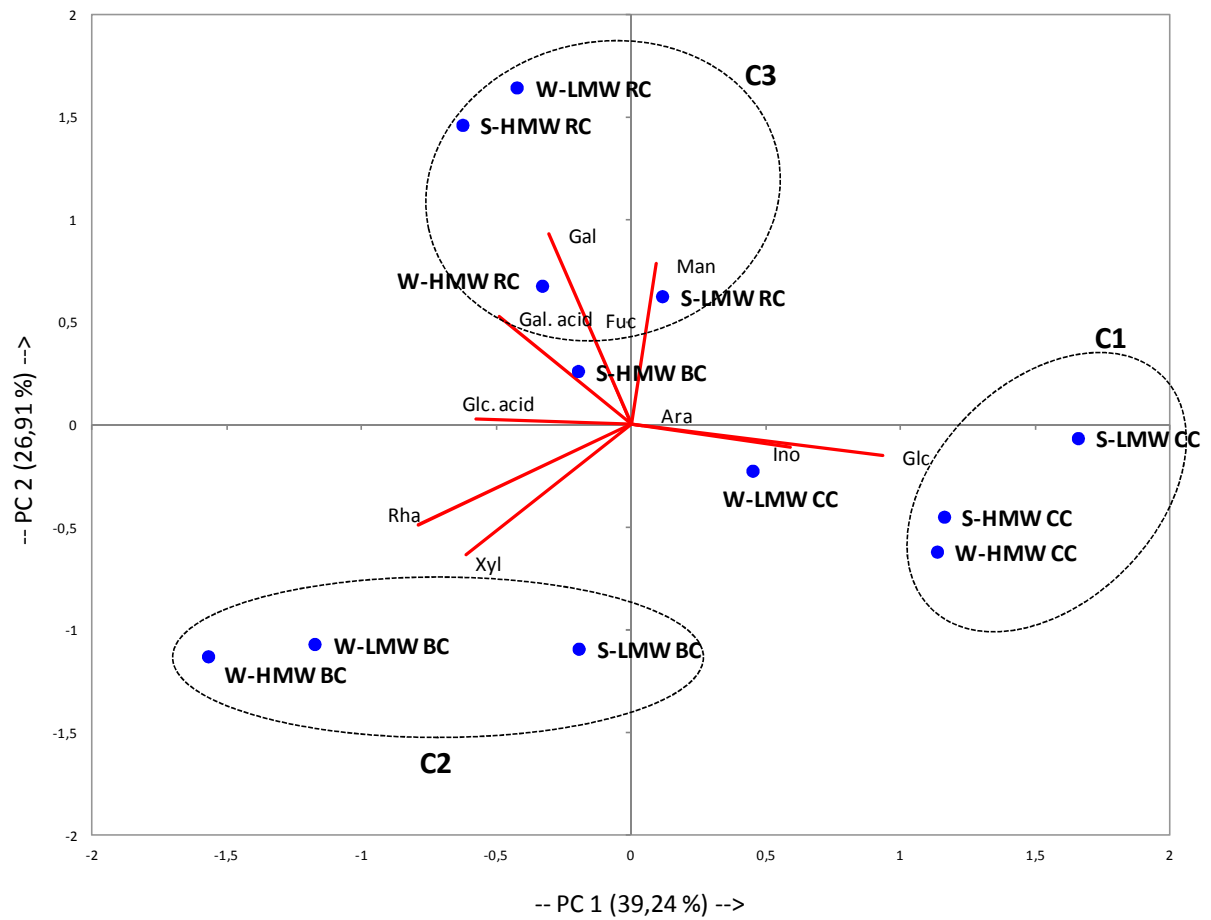
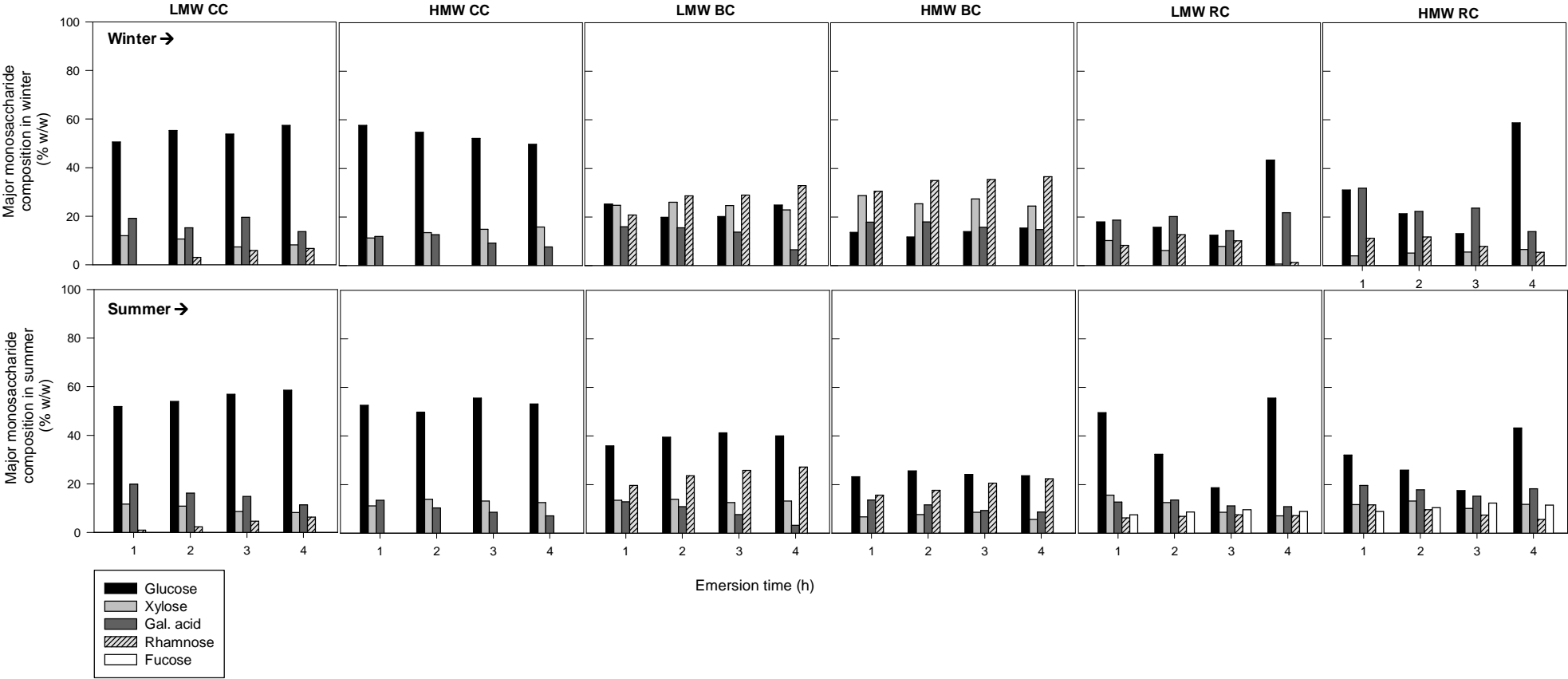


FIGURE 6

612



613

614

615

616

FIGURE 7