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Biochemical characterization of Extracellular Polymeric Substances extracted from an intertidal mudflat using a cation exchange resin.

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ABSTRACT

The biochemical characterization of Extracellular Polymeric Substances (EPS) excreted in a European intertidal mudflat (Marennes-Oléron Bay) was performed. Experiments were carried out for the first time \textit{in situ}, by using an improved extraction recently developed. This innovative procedure, using a cation exchange resin (Dowex), allows separating precisely different fractions of EPS, especially pure bound EPS. Moreover, it avoids the contamination of EPS fractions by residual and intracellular polymers, enabling to properly estimate polymeric contents in each fraction. The results were partly similar to conventional results described in the literature and the amount of colloidal carbohydrates (146µg/g of dry sediment) extracted by the Dowex method fitted well with different EPS estimation in
European mudflats. Colloidal carbohydrates were essentially composed of glucose (>50%), a carbon source rapidly consumed by the various communities in the sediment. Pure bound carbohydrates were composed of specific carbohydrates (28% rhamnose, 22% xylose). Residual fractions, considered as containing some refractory bound EPS and mostly other internal polymeric substances, presented a more varied composition rich in carbohydrates: galacturonic acid (20%), mannose (19.5%), glucose (19%), arabinose (15%), xylose (8%), galactose (7%).

Keywords: Extracellular compounds, biochemical characterization, biofilm, in situ quantification, benthic ecology

1. Introduction

The benthic biofilms which developed during the emerged periods in intertidal mudflats are widely studied for various reasons. Considering an ecological perspective, the main reason is to understand the influence of benthic biofilms in intertidal ecosystems, by analyzing their compositions and their changes, depending on environmental parameters. Numerous studies of the last decades have allowed determining their composition and highlighted the presence of microalgae (microphytobenthos), bacteria and fungi, tangled in a complex mixture of polymeric compounds that they produce (Frølund et al., 1996). These Extracellular Polymeric Substances (EPS) are rich in polysaccharides, proteins, proteoglycans, lipids and many other compounds expressed at different levels (Stoodley et al., 2002; Stal, 2003), related to the location or environmental conditions which affect both food web and primary production of this ecosystem (Underwood and Paterson, 2003). EPS are involved in the mobility system of
epipelic diatoms (Stal and Défarge, 2005) and can be used as carbon sources by the bacterial community (van Duyl et al., 1999; Hofmann et al., 2009). EPS also affect the microenvironment of biofilms by varying physico-chemical parameters like porosity or mechanical stability of the sediment (Orvain et al., 2003; Perkins et al., 2004; Spears et al., 2008). On the other hand, benthic EPS can present interesting structures and functions, which can be used in many biochemical fields. The extraction of sulfated polysaccharides for medicinal applications is one example (Witvrow et al., 1997). Many works have already proposed extraction protocols allowing the collection of particular EPS, having specific biochemical properties (Stats et al., 1999; de Brouwer et al., 2001; Azerado et al., 2003; Bellinger et al., 2005, Comte et al., 2006). All the data obtained have been each time criticized and authors have agreed that there was no universal extraction method for EPS. Recently, Takahashi et al. (2009) have optimized a protocol for EPS extraction and proposed an innovative method, using a cation exchange resin, to extract cultured diatoms EPS without any contamination by internal compounds. Furthermore, the method allowed the extraction of bound EPS, a fraction poorly studied and heavily contaminated in other previous studies (de Brouwer and Stal, 2004; Chiovitti et al., 2004).

The aim of the present investigation was to characterize the biochemical composition of EPS collected from benthic biofilms during emerged periods on a macrotidal bay (Marennes-Oléron Bay, France), using for the first time in situ the Dowex-resin method (Takahashi et al., 2009).

2. Methods

2.1. Intertidal mudflat samples

The mud samples used in this study were collected from Marennes-Oléron Bay (Atlantic Coast of France) in February 2008 (winter) at low tide (Fig. 1). Two hours after the beginning
of the emersion, sediment cores were sampled for three different squares, to take into account spatial heterogeneity. Sediment samples were collected using core diameter of 20 cm, and the top 1 cm was collected three times and pooled to give a main sediment core, for each square. After sampling, sediment was brought back on from the field by using a watercraft for an immediate EPS extraction on fresh sediments on the upper shore. The colloidal, bound and residual fractions were extracted through the Dowex-resin method then biochemical analyses were performed, all in triplicate.

2.2. 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 sulfate, heparin, fucoidan, 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 J&W Scientific column (30m, 0.32mm, 1µm) for Gas Chromatography-Mass Spectrometry analysis (GC/MS) was obtained from Agilent.

2.3. EPS Extraction in situ

The extraction was done immediately after sampling and sediment mixing (Takahashi et al. 2009). 20 mL of fresh mudflat was mixed with 20 mL of Artificial Sea Water (ASW 30 Practical Salinity Units) during 1 h in darkness at 4°C and then centrifuged at 3500 g and 4°C for 10 min. The supernatant (a) containing colloidal EPS was collected and stored at 4°C. 20 mL of ASW and 1 g of activated Dowex (Marathon C, activated in Phosphate Buffer Saline for 1 h in the dark) was added to the cap (b). The samples were mixed gently at 4°C for 1 h in the dark and then centrifuged at 3500 g and 4°C for 10 min. A supernatant containing the
bound EPS (c) and a cap containing intracellular and residual polymers (d) were obtained. The cap was then frozen. The residual polymers were extracted from the frozen samples, by sonication at 100W for 3 min on ice after resuspension in 20 mL in ASW.

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

2.4. EPS Composition

Total sugar content was determined using the phenol-sulfuric acid assay, developed by Dubois, using glucose as a standard (Dubois et al., 1956). Protein content was determined using the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) as a standard (Smith et al., 1985). Uronic acid content was determined using the meta-hydroxydiphenyl method (MHDP), using galacturonic and glucuronic acids as standards (Blumenkrantz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991). The sulfate content was measured by the Azure A (Jaques et al., 1968) and the Ba/Cl₂ gelation method (Craigie et al., 1984), using Dextran sulfate as a standard.

2.5. Sugar Characterization

Prior to carbohydrate characterization by GC/MS, EPS fractions were solubilized in 5 mL of ultra-pure water, dialyzed (6-8 KDa) and freeze-dried (Bellinger et al. 2005). EPS were then dissolved in 2M HCl at 50 mg/mL and heated at 90°C for 4 h. The preparation (which contained mostly carbohydrates monomers) was then freeze-dried and stored at -20°C. Analysis of the carbohydrate fractions were carried out by GC/MS using a Varian CP-3800
GC/Varian Saturn 2000 (Fig. 2). Operating conditions have been determined and optimized in the laboratory (data not shown). 400 µL of pyridine and 400 µL of BSTFA: TMCS (99:1) was added to 2 mg of purified polysaccharides. 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 1mL/min. The helium pressure was 8.8psi. 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 0min, 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.

3. Results and discussion
3.1. Type and composition of EPS

Despite the fact that common practice is to freeze sediments at -80°C to eliminate the consumption of EPS by bacteria, Takahashi’s extraction method focus on the use of fresh sediments to avoid cells lysis (Takahashi et al., 2009), which supports other studies concerning the contamination of EPS fractions by internal storage compounds, as glucans, proteins and chrysolaminaran (de Brouwer et al., 2001, Hanlon et al., 2006). The addition of Dowex resin to a classical procedure clearly defines pools of carbohydrates, depending on their properties and localization in the matrix complex (Bellinger et al., 2005; Abdullahi et al., 2006).

Overall, 1g of dry sediment is composed of 1618 µg of carbohydrates and 383 µg of proteins (Table 1-2). Although this concentration may seem low, EPS are extracted from crude samples. Different authors have shown that natural mudflats samples may contain large quantities of mineral impurities and salt (de Brouwer et al., 2001, Underwood and Paterson, 2003). The amounts of carbohydrates were slightly higher than those measured at the same
station in 1998 (Stal and Défarge, 2005). These authors had worked from the first 5 mm of sediment, which represent more accurately the microphytobenthic biofilm. Our approach (first 10 mm) could overestimate diatom EPS production due to the contamination from other sediment EPS sources (Perkins et al., 2003).

Colloidal fractions were rich in carbohydrates (±50/50 % LMW/HMW) (Table 1-2). LMW colloidal fractions could be compared to the low molecular weight exudates and HMW colloidal fractions EPS extracted by various authors (Abdullahi et al., 2006, Hanlon et al., 2006). The total amount of colloidal carbohydrate (neutral carbohydrates and uronic acids) given in Table 1 (146 µg.g⁻¹ sediment) fitted with common results described in literature: 50 to 5000 µg.g⁻¹ sediment. Similarly, the bound fractions were composed of carbohydrates (87 % LMW). The total amount of bound carbohydrates was closed to the concentrations of total colloidal carbohydrates (±113 µg.g⁻¹). This result would indicate that, in general, the colloidal and bound EPS are produced in close quantities in this benthic ecosystem. However, this amount of bound carbohydrates is lower than other amounts measured for European mudflats and suggests that our fractions were not contaminated by residual and internal carbohydrates. Colloidal and bound fractions did not contain proteins, in contrast to many previous works (de Brouwer et al., 2001; Underwood and Paterson, 2003; Hanlon et al., 2006, Hofmann et al., 2009). This lack seems to confirm that our EPS fractions were not contaminated by residual and internal storage polymers (Staats et al., 2000; Orvain et al., 2003).

Finally, the residual fractions were widely extracted compared to the EPS fractions (Table 2) and presented a complex composition especially because of the presence of proteins (22 %) and sulfated components (15 % of the total amount of carbohydrates) (Table 1). Our residual polymers found could be compared to the complex cell wall-associated and the intracellular polymers of diatoms, widely described in the literature (glucan, chrysolaminaran). Otherwise,
these residual fractions must also contain some refractory EPS that were not extracted with the Dowex-resin.

3.2. Model of Underwood & Smith

The colloidal EPS quantities measured in the Marennes-Oléron mudflat were compared to the predicted quantities given by the model of Underwood & Smith, which was used to determine the amount of colloidal carbohydrates produced in European mudflats (Underwood and Smith, 1998).

\[
\text{log (coll. carbohydrates content +1)} = 1.40 + 1.02 \times \text{log (Chl } a \text{ content +1)}
\]

\[r^2=64.6\%\]

The model was applied to the concentrations of Chlorophyll \( a \) (in average, 21.5 \( \mu \text{g Chl } a/g \text{ dw sediment} \) measured in situ during the sampling campaign. Considering the relationship and the \( r^2 \), the amount of colloidal carbohydrates extracted by the Dowex-resin was in accordance with the model, suggesting that the Dowex method allowed extracting in full the colloidal EPS.

3.3. Sugar characterization

GC-MS results indicated that the carbohydrate portions were formed of nine different types of monomer units (Fig. 3), including seven neutral sugars and two uronic acids. The colloidal EPS fractions had a high glucose content (>50 %), the bound EPS fractions were mainly composed of rhamnose, xylose, glucose, galacturonic acid and the residual polymeric fractions had a more varied composition in monosaccharides, including a greater unknown.

The monosaccharide distribution between LMW and HMW of the colloidal fractions was quite similar, although there were a few amount of rhamnose in LMW fractions (Fig. 3, A1-A2). Colloidal EPS fractions were mainly composed of glucose, which could explain why this fraction is easily consumable by heterotrophic bacteria in the extracellular medium (van Duyl
et al., 1999; Bellinger et al., 2005; Hofmann et al., 2009). Our results were close to previous works, which showed the predominance of glucose (50 %), galactose, xylose (15 %) or galacturonic acid (15 %) in colloidal fractions extracted by the same way (Abdullahi et al., 2006; Hanlon et al., 2006). In contrast to previous works where bound fractions were extracted, glucose is not the main saccharide (less than 20 %) of bound EPS fractions (Fig. 3, B1-B2). The content of specific sugars in these fractions can be better estimated and bound EPS were mainly composed of rhamnose (28 %), xylose (22 %) and galacturonic acid (18 %). The accurate composition of bound EPS, enriched in deoxy and specific sugars, is very important for understanding the functional role of bound EPS. Deoxy sugars can promote biostabilisation of sediments (Zhou et al., 1998; Giroldo et al., 2003) through their surface active properties. Deoxy sugars can also influence the hydrophobic character of EPS, playing a role on the adhesion of EPS to sediment or on the regulation of desiccation and salinity (Spears et al., 2008). However, it was surprising that fucose has not been highlighted although the GC-MS method allowed its detection. It is therefore possible that the lack of fucose was linked to the environmental conditions or the physiological state and the quantity of benthic diatoms forming the benthic biofilm during winter (Stal and Défarge, 2005; Bellinger et al., 2009). The presence of inositol (−myo) is significant since no author has highlighted it. Inositol is involved in the structural basis for a number of secondary messengers in eukaryotic cells and is a major growth factor for many-organisms, especially for heterotrophic bacteria. Residual fractions were mainly composed of polysaccharides rich in glucose (derived from β-1,3-linked glucan or chrysolaminaran) and mannose, rhamnose, xylose (Fig. 3, C1-C2). It is important to note that a portion of these sugars must come from refractory bound EPS. On the whole, our results confirmed the relevance and the effectiveness of Takahashi’s method for in situ experiments. Bound fractions were biochemically different from the two other fractions, thanks to the presence of large amounts of deoxy sugars and uronic acids. In
this way, it can be supposed that the levels of rhamnose, mannose or galacturonic acid played a role during the development of the microphytobenthic biofilm by increasing binding forces or enhancing the incorporation of water. The surprising lack of fucose has been correlated to the sampling period. The presence of inositol was detected for the first time in situ. This sugar is commonly used for GC/MS analysis (internal standard) and this could explain why it was not identified as a component of EPS. Significant amounts of this growing factor for bacteria were measured in the colloidal fraction, a fraction known as being a direct nutrient source for the heterotrophic bacteria. Finally, it would be interesting to extract and characterize in situ the same EPS fractions depending on environmental conditions.

Acknowledgements

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References


Table 1 Composition (µg/g of dry sediment) of the different fractions extracted by the Dowex method from the Marennes-Oléron mudflat.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Neutral carbohydrate content</th>
<th>Uronic acid content</th>
<th>Sulfate content</th>
<th>Protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW colloidal</td>
<td>59 ± 8</td>
<td>23 ± 9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HMW colloidal</td>
<td>57 ± 16</td>
<td>7 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LMW bound</td>
<td>76 ± 8</td>
<td>22 ± 10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HMW bound</td>
<td>11 ± 2</td>
<td>4 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LMW residual</td>
<td>413 ± 79</td>
<td>112 ± 38</td>
<td>0</td>
<td>180 ± 72</td>
</tr>
<tr>
<td>HMW residual</td>
<td>530 ± 155</td>
<td>304 ± 172</td>
<td>204 ± 66</td>
<td>203 ± 55</td>
</tr>
</tbody>
</table>

± : deviations were calculated from the heterogeneity of the different sampling squares and not from the true replicates of the biochemical analysis (<5%)
Table 2 Distribution of carbohydrates in the three fractions of EPS.

<table>
<thead>
<tr>
<th>% (w/w)</th>
<th>Fraction Ratio</th>
<th>Low Molecular Weight</th>
<th>High Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal carbohydrates</td>
<td>9</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>Bound carbohydrates</td>
<td>7</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>Residual carbohydrates</td>
<td>84</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>100*</td>
<td>43</td>
<td>57</td>
</tr>
</tbody>
</table>

* (1618µg/g of dry sediment)
Fig. 1. Station where samples of surficial intertidal sediment were collected, two hours after the beginning of emerged period.

Fig. 2. GC-MS chromatogram of carbohydrates detected in an HMW bound fraction. Ionization: Electronic Impact (EI). Target ion: 40-650 m/z.

Fig. 3. Monosaccharide composition of the different EPS fractions collected on the Marennes-Oléron mudflat (% of the carbohydrate content) after 2 hours of emersion. Ionization: Electronic Impact (EI). Target ion: 40-650 m/z. The variability within true sample replicate was less than 5%. (White): Unknown, undetermined on GC/MS.
FIGURE 2
FIGURE 3