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Seasonal variations of the composition of microbial biofilms in sandy tidal flats: Focus of fatty acids, pigments and exopolymers

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4 **Seasonal variations of the composition of microbial biofilms in sandy tidal**
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6 **flats: focus of fatty acids, pigments and exopolymers**
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43 pigments.
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1
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4 **Abstract**
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6 Biofilms, or microbial mats, are common associations of microorganisms in tidal flats;
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8 they generally consist of a large diversity of organisms embedded in a matrix of Extracellular
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10 Polymeric Substances (EPS). These molecules are mainly composed of carbohydrates and
11
12 proteins, but their detailed monomer compositions and seasonal variations are currently
13
14 unknown. Yet this composition determines the numerous roles of biofilms in these systems.
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16 This study investigated the changes in composition of carbohydrates in intertidal microbial
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18 mats over a year to decipher seasonal variations in biofilms and in varying hydrodynamic
19
20 conditions. This work also aimed to assess how these compositions are related to microbial
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22 assemblages. In this context, natural biofilms whose development was influenced or not by
23
24 artificial structures mimicking polychaete tubes were sampled monthly for over a year in
25
26 intertidal flats of the Chausey archipelago. Biofilms were compared through the analysis of
27
28 their fatty acid and pigment contents, and the monosaccharide composition of their EPS
29
30 carbohydrates. Carbohydrates from both colloidal and bound EPS contained mainly glucose
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32 and, to a lower extent, galactose and mannose but they showed significant differences in their
33
34 detailed monosaccharide compositions. These two fractions displayed different seasonal
35
36 evolution, even if glucose accumulated in both fractions in summer; bound EPS only were
37
38 affected by artificial biogenic structures. Sediment composition in fatty acids and pigments
39
40 showed that microbial communities were dominated by diatoms and heterotrophic bacteria.
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42 Their relative proportions, as well as those of other groups like cryptophytes, changed
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44 between times and treatments. The changes in EPS composition were not fully explained by
45
46 modifications of microbial assemblages but also depended on the processes taking place in
47
48 sediments and on environmental conditions. These variations of EPS compositions are likely
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50 to alter different ecosystem processes such as biostabilisation or pollutants trapping.
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Introduction

In coastal soft-bottom areas, a large diversity of microbial mats exists; their composition depends on environmental conditions such as salinity, tidal height and duration of exposure, light, grain size, and organic matter input (Brotas et al., 1995; Franks and Stolz, 2009; Paterson and Hagerthey, 2001). Additionally, they are sensitive to anthropic actions such as eutrophication or antibiotic release in the environment (Lubarsky et al., 2012; Sawall et al., 2012). These biofilms are often very productive and they also provide different ecosystem services, such as nutrient recycling, degradation of pollutants and sediment stabilisation, and they are essential for the recruitment of numerous benthic organisms (Decho, 2000).

Biofilms consist of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which are produced by the different organisms of the biofilm. These EPS are chemically diverse molecules but are mainly composed of carbohydrates and protein, and contain very few lipids. EPS have various roles both for microorganisms: they protect from pollutants and desiccation (Decho, 1990; Potts, 1994) and they allow locomotion, especially for diatoms, and attachment to sediment (Wotton, 2004). They are also important for coastal systems, as they are for instance responsible for sediment stabilisation in intertidal areas (Decho, 1990).

Because their composition varies depending on their environment, microbial mats are useful as proxies of environmental conditions. Therefore, various markers have been developed to characterise the microbial composition of biofilms, and, more recently, their EPS composition. Quantification of carbohydrates and proteins has been a first step, and GC-MS and LC-MS (gas and liquid chromatography coupled with mass spectrometry) now allow a further analysis of these molecules. Carbohydrates in particular have been investigated as they are crucial for sediment stabilisation by biofilms (de Brouwer et al., 2005); these

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4 molecules have been characterised through their monosaccharide composition, which has
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6 been shown to vary according to organisms and environmental conditions (de Brouwer and
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8 Stal, 2002; Hanlon et al., 2006; Underwood et al., 2004).
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11 Due to the numerous roles of EPS in coastal systems, and given that these roles
12
13 depend on EPS composition, characterising EPS composition and variations is an important
14
15 challenge to understand the provision of ecosystem services by microbial mats in varying
16
17 environments. Therefore, this study was designed to test the hypothesis that the EPS
18
19 composition of biofilms: (1) varies seasonally and is influenced by other environmental
20
21 conditions; and (2) does not only depend on the microorganisms forming the microbial mats.
22
23 Biofilms were followed over the year on-field in an intertidal area, in three experimental
24
25 conditions. Artificial tubes mimicking polychaete tubes were used to modify biofilm
26
27 development, as such tubes influence both microbial expansion and EPS production
28
29 (Passarelli et al., 2012). Biofilms were sampled monthly and the microbial assemblage was
30
31 characterised by their fatty acid and pigment contents. EPS were characterised by the
32
33 monosaccharide composition of their carbohydrates.
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41 **Material and methods**

42 **Study site**

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45 Experiments were carried out from March 2011 to April 2012 in the Chausey
46
47 archipelago (France) within the Normand-Breton Gulf (English Channel), which is
48
49 characterized by a megatidal regime (tidal range up to 14 m). The Chausey archipelago
50
51 includes 1500 ha of soft sediment tidal flats.
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56 **Experimental design**

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4 Twelve cylindrical benthic chambers of 300 mm inner diameter and 60 mm depth
5
6 were placed in the intertidal sandy area of Chausey archipelago (Normand-Breton Gulf,
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8 France). Solid PVC cylindrical rods were fixed to the bottom of each benthic chamber,
9
10 simulating tubes of large polychaete “builder” species, such as *Lanice conchilega* or *Melinna*
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12 *cristata* (5 mm diameter, 95 mm length). Three treatments were used (4 replicate chambers
13
14 per treatment); a control treatment (Ctrl), without tubes; then 2 test treatments with varying
15
16 densities of evenly spaced tubes: a low density (LD) treatment (637 tubes·m⁻²) and a high
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18 density (HD) treatment (2505 tubes·m⁻²). The LD treatment potentially destabilised the
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20 sediment (i. e. initiated interactive flow; Friedrichs et al., 2000), whereas the HD treatment
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22 most likely had a stabilising effect on it (i. e. initiated skimming flow; Friedrichs et al., 2000).
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24 These densities were both within the range of natural densities for polychaete tubes (up to
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26 5000 ind·m⁻²; e.g. Friedrichs et al. 2000 for *Melinna cristata*).
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32 Before positioning the chambers in the field, they were filled with defaunated sand
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34 (washed with 1 mol·l⁻¹ HCl, frozen and thawed) collected at the study site (medium sand,
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36 median grain size between 260 and 300 µm). They were then placed into the intertidal area on
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38 21 March 2011, with a 2 m gap between chambers. Sampling was carried out approximately
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40 every month (14 times in the experiment, depending on tidal height and accessibility of the
41
42 study site) from the beginning of the experiment to 8 April 2012.
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46 Sampling locations within the chambers were randomly chosen for each sampling
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48 time, taking care that a single location was never sampled twice in a 3-month period.
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51 Visual observation of the plots confirmed that any cavities created by sampling were
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53 filled by natural sediment within 24 h, after which no trace of the sampling was apparent.
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58 **Sampling and measurements**

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Fatty acids composition of the sediment

One sample was taken from each benthic chamber at every sampling date (2 cm inner diameter cut off syringe, 1 cm depth). Sediment cores were frozen and transported back to the laboratory where they were freeze-dried, and stored at -26 °C. 400 to 500 mg of sediment were weighted and used for the analysis. Fatty acid (FA) analysis was performed following the modified method of Bligh & Dyer (1959) as described in Mortillaro et al. (2011). Before extraction, an internal standard (FA 23:0) was added to every sample for quantification purpose. Lipids were extracted with a 20 min ultrasonication in a mixture of distilled water, chloroform and methanol in ratio 1:1:2 (v:v:v). Lipids were concentrated under N₂ flux, and saponified, in order to separate FAs, with a mixture of NaOH (2 mol·l⁻¹) and methanol (1:2, v:v) at 90 °C during 90 min. Saponification was stopped with hydrochloric acid. FAs were then derivatized with BF₃-methanol (boron-trifluoride methanol) at 90 °C during 10 min and kept frozen in chloroform. Just before analysis, samples were dried under N₂ flux and transferred to hexane. One µl of the mixture was injected in a gas chromatograph (GC, Varian CP-3800 equipped with flame ionization detector), which allowed separation and quantification of FAs. Separation was performed with a Supelco OMEGAWAX 320 column (30 m × 0.32 mm i.d., 0.25 µm film thickness) with He as carrier gas. The following temperature program was used: 60 °C for 1 min, then raise to 150 °C at 40 °C·min⁻¹ (held 3 min), then raise to 240 °C at 3 °C·min⁻¹ (held 7 min). Peaks of FAs were identified by comparison of the retention time with analytical standards (Supelco™ 37, PUFA-1 Marine Source, and Bacterial Mix, Supelco Inc., USA) and analysis of the sample in a gas chromatograph coupled to mass spectrometer (GC-MS, Varian 450GC with Varian 220-MS).

FAs are designated as X:YωZ, where X is the number of carbons, Y the number of double bonds and Z the position of the ultimate double bond from the terminal methyl.

Pigment composition of the sediment

Three samples were taken from each benthic chamber at every sampling date (2 cm inner diameter cut off syringe, 1 cm depth), and pooled. Samples were kept frozen at -26 °C until analysis. Pigments were extracted from a sub-sample of approximately 0.2 g of freeze-dried sediment with 2 ml of 95 % cold buffered methanol (2 % ammonium acetate) for 15 min at -20 °C, in the dark. Samples were sonicated for 30 s at the beginning of the extraction period. Extracts were filtered with Whatman membrane filters (0.2 mm) immediately before HPLC analysis. Pigment extracts were analysed using a Shimadzu HPLC comprised of a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP), a photodiode array (SPD-M10AVP) and a fluorescence detector (RF-10AXL). Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Supelcosil, 25 cm long, 4.6 mm in diameter, and 5 µm particles). The solvents used were 0.5 mol·l⁻¹ ammonium acetate in methanol and water (85:15, v:v), acetonitrile and water (90:10, v:v), and 100 % ethyl acetate. The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 ml·min⁻¹ and an injection volume of 100 µl. Identification and calibration of the HPLC peaks was confirmed with chlorophyll *a*, chlorophyll *b* and b-carotene standards from Sigma and chlorophyll *c*, fucoxanthin, diadinoxanthin, diatoxanthin, lutein, zeaxanthin, and pheophytin *a* standards from DHI. Pigments were identified by the absorption spectra and retention times and the concentrations calculated from the signals in the photodiode array (chlorophylls and carotenoids) or fluorescence detector (pheophorbides and pheophytins). As samples were not frozen in situ but transported in the dark before being frozen, diatoxanthin had time to revert to diadinoxanthin. Therefore, concentrations of diatoxanthin and diadinoxanthin were added and analysed together as xanthophyll concentration.

EPS composition in monosaccharides

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4 Three samples were taken from each benthic chamber at every sampling date (2 cm
5 inner diameter cut off syringe, 1 cm depth), pooled onto one sample per chamber, and frozen
6 until analysis. Monosaccharide composition of carbohydrates was determined using gas
7 chromatography (GC), following a method adapted from Pierre et al. (2010). Briefly, EPS
8 were extracted, and carbohydrates decomposed into monosaccharides with acidic hydrolysis.
9 Sugars were thereafter silylated and separated with GC.
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18 Colloidal EPS were extracted by rotating the samples in artificial sea water ($30 \text{ g}\cdot\text{l}^{-1}$)
19 1h at $4 \text{ }^\circ\text{C}$. Bound EPS were thereafter recovered by adding resin (Dowex Marathon C,
20 sodium form, Sigma) to the remaining sediment and performing a second extraction. Both
21 fractions were dialysed against distilled water (12 – 14 kDa) and freeze-dried.
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28 EPS were dissolved in $2 \text{ mol}\cdot\text{l}^{-1}$ HCl, and heated 4 h at $90 \text{ }^\circ\text{C}$. The solution then
29 mainly contained monosaccharides, and was freeze-dried again.
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32 Silylation was performed with a mixture of BSTFA:TMCS (N,O-
33 bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane, 100:1, Sigma) and pyridine
34 (1:1, v:v), 2 h at room temperature. After the silylation, $1 \mu\text{l}$ of the sample was injected in GC
35 (GC, Varian CP-3800 equipped with flame ionization detector), which allowed separation and
36 relative quantification of sugars. Separation was performed with an Agilent Technologies VF-
37 1701ms column ($30 \text{ m} \times 0.32 \text{ mm i.d.}$, $0.25 \mu\text{m}$ film thickness) with He as carrier gas. The
38 following temperature program was used: start at $150 \text{ }^\circ\text{C}$, raise to $200 \text{ }^\circ\text{C}$ at $7 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ (held
39 5 min). Injector temperature was set at $250 \text{ }^\circ\text{C}$. Peaks of monosaccharides were identified by
40 comparison of the retention time with analytical standards (rhamnose, fucose, xylose,
41 mannose, galactose, glucose, scyllo- and myo-inositol, galacturonic and glucuronic acid),
42 which had been prepared (silylation and injection only) as samples.
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58 Carbohydrate and protein concentrations in EPS were measured by colorimetry
59 following methods from Lubarsky et al. (2010).
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7 **Statistical analyses**
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9 Non-parametric statistics under the R statistical framework were used for univariate
10 analyses as data violated assumptions of homogeneity of variance and normality. Differences
11 between treatments were assessed by Kruskal-Wallis tests (KW), while differences between
12 sampling time for each treatment were assessed by Friedman tests (considering data from
13 each chamber as paired data).
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20 Non-metric MultiDimensional Scaling (nMDS) analyses (500 iterations) were
21 performed with similarity matrix constructed with Bray Curtis distances for FAs, pigments
22 and sugars. These graphical representations show similarity between samples: the closer the
23 symbols are in the representation, the closer the characteristics of the samples are. 2-factor
24 ANOSIMs (crossed factors: Time and Treatments) were then used to assess the difference
25 between assemblages from different sampling time (Primer 5 software, 10000 iterations), and
26 variables responsible for such differences were investigated with SIMPER analyses.
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37 Multiple Factor Analysis (MFA) was performed to compare chambers at different
38 sampling time using FA, pigment and sugar datasets. MFA studies several groups of variables
39 defined on the same set of individuals. The core of the method is a factor analysis applied to
40 the whole set of variables, in which each group of variables is weighted so that no group of
41 variables become dominant over the others (Escofier and Pagès, 1994). Data were scaled to
42 unit variance.
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54 **Results**
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56 **Composition of the microbial community**
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58 Fatty acid contents
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4 Overall compositions of microbial communities and organic matter in sediment was
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6 assessed through their fatty acid (FA) content. Quantitative data (Fig. S1) showed
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8 accumulation of FAs in sediment in early August and late September. FAs found in our
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10 sediment samples are listed on table S1. Most abundant FAs were saturated FAs (SFAs) 14:0,
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12 16:0, 18:0, mono-unsaturated FA (MUFA) 16:1 ω 7 and polyunsaturated FA (PUFA) 20:5 ω 3,
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14 suggesting a large abundance of diatoms in these biofilms. Moreover, branched FAs (BFAs)
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16 reached a 2 % relative contribution, showing the presence of heterotrophic bacteria.
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20 Data showed a dramatic change in FA composition of sediment through time (Fig. 1;
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22 ANOSIM: $R = 0.767$, $p < 0.001$). Samples from two following months were significantly
23
24 different one from each other's, except in winter where samples were similar (ANOSIM post-
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26 hoc pairwise tests, Table 1). SIMPER analysis showed that FAs 16:1 ω 7 and 20:5 ω 3, as well
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28 as saturated FAs (SFAs; mainly 14:0, 16:0 and 18:0) were responsible for the differences
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30 between time groups. Namely, proportions of 16:1 ω 7 and 20:5 ω 3 varied significantly through
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32 time in every treatment (Friedman tests, $p < 0.001$); they were present in higher proportions in
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34 April, May, at the beginning of August, in some samples of November and from January to
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36 March 2012, compared to other months. On the contrary, the ratio between saturated and
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38 polyunsaturated FAs (SFA/PUFA ratio) was lower in these months.
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43 Samples were also significantly different between treatments (ANOSIM, $R = 0.284$,
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45 $p < 0.001$; ANOSIM post-hoc pairwise tests Table 2), although the time effect seemed
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47 predominant (Fig. 1). Main differences were explained by MUFA 16:1 ω 7, PUFA 20:5 ω 3 and
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49 SFA 18:0, which were more abundant in Ctrl treatments compared to others; by SFA 14:0
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51 which was more abundant in both treatments with tubes; and by SFA 16:0 which accumulated
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53 more in LD chambers.
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60 Pigment contents

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4 Overall composition of the photosynthetic communities in sediment was assessed
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6 through their pigment content. Total pigment concentrations (Fig. S1) showed accumulation
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8 in summer (LD treatments) or after summer (other treatments). Sediment samples contained
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10 mainly chlorophyll *a*, fucoxanthin, alloxanthin and chlorophyll *c* (Table S2), showing the
11
12 preponderance of diatoms and cryptophytes.
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15 Pigment composition of sediment also changed through time (Fig. 2; ANOSIM:
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17 $R = 0.393$, $p < 0.001$). Significant differences between two following month mostly appear in
18
19 late summer, at the beginning of autumn and in winter (ANOSIM post-hoc pairwise tests,
20
21 Table 1). Regardless of the sampling time, chlorophyll *a* accounted for approximately 50 % of
22
23 pigments in sediment, while proportions of fucoxanthin and alloxanthin exhibited significant
24
25 differences among time (Friedman tests, $p > 0.05$ for chlorophyll *a* and $p < 0.05$ for
26
27 fucoxanthin and alloxanthin). Pigments contributing highly to dissimilarities between seasons
28
29 were chlorophylls *a* and *c*, which were in higher proportions in autumn and winter, and
30
31 chlorophyllide *a* and xanthophylls, which were in higher proportions in spring and summer.
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35 Pigment composition of sediment changed weakly between treatments (ANOSIM:
36
37 $R = 0.08$, $p = 0.016$): chambers with tubes had significantly different compositions compared
38
39 to control chambers (ANOSIM post-hoc pairwise tests, Table 2). Chlorophyllide *a* was indeed
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41 in lower proportion and alloxanthin in higher proportion in Ctrl chambers compared to other
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43 chambers.
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51 **EPS**

52 General composition

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56 EPS total concentrations in both carbohydrates and proteins showed variations through time,
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58 with accumulation of EPS at the beginning of summer (Fig. S3). Glucose was the most
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60 abundant sugar, followed by mannose and galactose (Table S3). Glucose percentages varied
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4 significantly through time in each treatment, for colloidal and bound fractions (Friedman
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6 tests, $p < 0.001$), with highest contributions in summer.
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10 11 EPS fractions 12

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14 Colloidal and bound fractions had slightly different monosaccharide compositions
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16 (ANOSIM: $R = 0.083$, $p < 0.001$). SIMPER analysis showed that 4 sugars explained more
17
18 than 70 % of dissimilarities between fractions: glucose was more abundant in colloidal EPS
19
20 while mannose, xylose and scyllo-inositol were more abundant in bound EPS.
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22

23 Therefore, the analysis was made separately for colloidal and bound EPS fractions.
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28 29 Colloidal EPS 30

31 Monosaccharide composition of colloidal EPS changed through time, with significant
32
33 differences between sampling months (Fig. 3; ANOSIM: $R = 0.375$, $p < 0.001$). Periods of
34
35 variations were distributed along the year (ANOSIM post-hoc tests, Table 1). Sugars
36
37 explaining most dissimilarity between sampling times were the most abundant: glucose,
38
39 mannose and galactose, as well as scyllo-inositol when comparing winter months with other
40
41 sampling times.
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45 No significant differences between treatments were found (ANOSIM, $R = 0.049$,
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47 $p = 0.068$).
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52 53 Bound EPS 54

55 Monosaccharide composition of bound EPS changed through time, with significant
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57 differences between sampling months (Fig. 4; ANOSIM, $R = 0.376$, $p < 0.001$). Significant
58
59 evolution of bound EPS composition was mainly found between August and February (Table
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4 1). Sugars explaining most dissimilarity between sampling times were also glucose, mannose
5
6 and galactose.
7

8
9 Bound EPS composition in monosaccharides also changed slightly between treatments
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11 (ANOSIM: $R = 0.079$, $p = 0.017$): Ctrl and LD had significantly different compositions
12
13 compared to HD chambers (ANOSIM post-hoc pairwise tests, Table 2).
14
15

16 17 18 **Multi-marker approach** 19

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21 Results of MFA combining datasets of FAs, pigments, colloidal and bound EPS are
22
23 displayed in Fig. 8. Glucose and galactose percentages in both EPS fractions contribute most
24
25 to the first axis, while pigments β -caroten, xanthophylls, pheophytin, percentage of myo-
26
27 inositol in bound EPS, and FA 16:1 ω 7 contribute most to the second axis. Results showed
28
29 correlations between different types of variables (Fig. 5.A); for instance, pigment pheophytin
30
31 *a* was associated with most of branched FAs. Combination of the four datasets allowed the
32
33 segregation of samples from different months along a clear seasonal cycle, with a specific
34
35 combination of biomarkers for each season (Fig 5.B).
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42 **Discussion** 43

44 **EPS composition over the course of the experiment** 45

46 Composition of carbohydrates in colloidal and bound EPS 47

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50 EPS were divided in two fractions according to their extraction protocol: the colloidal
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52 fraction was extracted with water only whereas bound EPS were collected with a strong
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54 cationic resin (Takahashi et al., 2009). This resin traps the cations maintaining the links
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56 between bound EPS, therefore allowing their release in the water. Our results demonstrate that
57
58 these two EPS fractions are chemically different, as their monosaccharide general
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4 compositions are significantly different. These two compositions of colloidal and bound EPS
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6 do not change simultaneously along the year (Table 1), suggesting that their control is specific
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8 to each EPS fraction. These different compositions are consistent with literature data showing
9
10 that both fractions have different production and degradation pathways (Staats et al., 1999;
11
12 Underwood and Paterson, 2003).
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16 Most abundant sugars were the same, but in different proportions, in both fractions.
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18 EPS carbohydrates consisted mostly in glucose, which always reached percentages over 50 %.
19
20 Colloidal EPS contained more glucose than bound EPS, which is consistent with literature
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22 data (Hofmann et al., 2009; Pierre et al., 2010; Taylor et al., 1999). Yet, Pierre et al. (2010)
23
24 obtained lower percentages of glucose, especially in bound fractions where percentage reached
25
26 20 % of total monosaccharide only. This difference could originate from a different producing
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28 community, for instance epipelagic assemblages instead of epipsammic ones, as they analysed
29
30 mud instead of sand in our study. Also, our sampling protocol, which includes a freezing step,
31
32 might prompt release of intracellular material, especially of some chrysolaminarin, which is
33
34 an intracellular storage glucan in diatoms, mainly composed of glucose (Chiovitti et al., 2004;
35
36 Underwood et al., 2004). Other abundant monosaccharides were mannose and galactose,
37
38 which is consistent with observation made on *Cylindrotheca closterium* cultures (Underwood
39
40 et al., 2004). Yet, the large amounts of glucose, mannose and galactose present in our
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42 sediment samples suggest that organic matter was mostly fresh, as these three sugars are
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44 particularly labile (Oakes et al., 2010).
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50 Variations in EPS compositions

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53 Both bound and colloidal carbohydrates displayed seasonal variations in their
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55 monosaccharide composition. Glucose was mainly responsible for seasonal differences; it
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57 accumulated in summer and was recorded in lower proportions in other seasons. Higher
58
59 percentages of glucose in EPS have been correlated with photosynthetic production (Bellinger
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4 et al., 2005; de Brouwer and Stal, 2001; Hanlon et al., 2006). Also, in the studied sediments,
5
6 the increase in glucose proportion in summer may originate from the degradation of
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8 intracellular chrysolaminarin, released in the environment by senescent diatoms. Other
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10 seasons displayed a higher diversity of sugars, which could be explained by the preferential
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12 degradation of glucose by heterotrophic bacteria (Bellinger et al., 2005; Hofmann et al.,
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14 2009): particularly, fucose, rhamnose and galactose are known to be more difficult for them to
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16 assimilate than glucose (Girollo et al., 2003). Finally, nutrient concentrations in pore water
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18 and light exposure of biofilms also vary seasonally, and both factors have been demonstrated
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20 to alter EPS carbohydrate composition (de Brouwer and Stal, 2002; Underwood et al., 2004),
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22 potentially strengthening the effects of photosynthesis and EPS degradation in shaping
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24 carbohydrate composition.
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30 Colloidal EPS compositions were not influenced by experimental conditions, while the
31
32 composition of bound carbohydrates appeared to vary between treatments: chambers with
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34 high tube density had different sediment composition compared to others chambers. Such
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36 effect might result from a direct influence of hydrodynamics on EPS production, or from an
37
38 indirect influence of tubes on benthic assemblages, or from a combination of both. As
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40 microbial assemblages and bound EPS compositions do not display similar temporal
41
42 evolutions (Table 1), a direct effect of hydrodynamics on the production or degradation of
43
44 bound EPS is a likely hypothesis. With such mechanisms, microorganisms could better adapt
45
46 their adhesion to the local current conditions. For instance, desoxy-monosaccharides like
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48 rhamnose and pentoses like xylose are able to promote adhesion to sediment because of their
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50 hydrophobicity (Bellinger et al., 2009).
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54 Functionality of different sugars

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57 Pierre et al. (2010) revealed the presence of myo-inositol in carbohydrates of marine
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59 sediment; this sugar is crucial for the functioning of many eukaryotic cells and is also a
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4 growth factor for various organisms. Our sediment samples also contained this sugar, and
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6 additionally its stereoisomer: scyllo-inositol. To our knowledge, this monosaccharide has
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8 never been found in EPS carbohydrates. In diatoms, it may originate from myo-inositol after
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10 transformation by the myo-inositol deshydrogenase enzyme (Gross and Meyer, 2003), which
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12 may explain its presence in the biofilm. Also, this monomer was an important sugar in
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14 separating winter from other seasons, which might be explained by variations in the
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16 functioning of this enzyme, although its role is still under investigation (Gross and Meyer,
17
18 2003). In soils, scyllo-inositol is associated to phosphate in scyllo-inositol hexakisphosphate,
19
20 which forms an important part of the organic phosphorus content (Turner et al., 2005).
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25 When studying different mudflats, de Brouwer et al. (2003) showed that EPS mainly
26
27 originated from refractory dissolved organic matter. This is probably not the case here, as
28
29 monosaccharide composition revealed a high proportion of glucose in every sample. Sugar
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31 composition can therefore be interpreted in terms of functionality for microorganisms. For
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33 instance, uronic acids have acidic groups which provide sites for sediment – organism
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35 interactions, so that they facilitate the fixation of cells to sediment (Bellinger et al., 2009).
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37 Also, deoxy-sugars such as fucose and rhamnose, and pentoses seem to promote
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39 biostabilisation of the sediment by enhancing the hydrophobicity of the biofilm (Bellinger et
40
41 al., 2009; Zhou et al., 1998). Finally, differences in monosaccharide composition have been
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43 shown to promote the development of different bacterial communities, depending on the
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45 ability of different bacterial groups to degrade these carbohydrates (Taylor et al., 2013).
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47 Changes in EPS composition and bacterial population will in turn provide recruitment cues
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49 for diverse organisms and can therefore facilitate or hinder the resilience of ecosystem
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51 engineers (Nelson et al., 2013).
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60 **EPS and microbial communities**

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Fatty acid biomarkers

In the studied environment, external factors varying seasonally such as temperature, photoperiod, and hydrodynamics have a strong structuring influence on EPS composition. EPS composition is also impacted by the composition of producing assemblages (Decho, 1990), which were in the present work characterised by their fatty acid (FA) and pigment compositions. FAs ubiquitous to all living organisms, but most taxonomic or functional groups are characterised by a given association of some FAs. For instance, branched FAs are only produced by bacteria (Arts and Wainman, 1999). Description of FA content of sediment provides, together, information on taxonomic or functional groups and on the degradation state of organic matter. The increase of SFA percentages, and of its ratio to PUFAs, demonstrates an accumulation of degraded OM in sediments in summer and autumn (Grossi et al., 2006; Sun et al., 1997). On the other hand, spring and winter months, as well as the beginning of August, are characterised by higher MUFA and PUFAs percentages; in particular, MUFA 16:1 ω 7 and PUFA 20:5 ω 3 (or EPA for eicosapentaenoic acid) relative contributions showed a drastic increase in these months. These FAs are mainly produced by microphytobenthos in these environments, especially diatoms, dinoflagellates and cyanobacteria (Arts and Wainman, 1999; Volkman et al., 1980). Yet the association of MUFA 16:1 ω 7 and PUFA 20:5 ω 3 together with high amounts of SFAs 14:0 and 16:0 suggest a dominance of diatoms (Dijkman et al., 2010).

Pigment biomarkers

Pigments were used to characterise the abundance and taxonomic composition of microphytobenthic communities. The abundance of fucoxanthin, together with chlorophylls *a* and *c*, is consistent with results of FA analysis in demonstrating the prevalence of diatoms in this environment (Jeffrey et al., 1997). Also, the presence of alloxanthin shows the occurrence of cryptophytes (Jeffrey et al., 1997). Proportions of chlorophylls *c* and fucoxanthin displayed

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4 a seasonal pattern, being higher in autumn and winter compared to spring and summer; on the
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6 contrary, chlorophyllide *a* showed a reversed pattern. This last pigment reveals the occurrence
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8 of senescent diatoms (Jeffrey et al., 1999), which suggests a higher predation rate in these
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10 months. Also, the occurrence of chlorophyllide *a* might be related to an exogenous supply of
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12 pigment. Xanthophylls displayed the same seasonal pattern than chlorophyllide *a*, which can
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14 be explained by their role in the xanthophyll cycle, which act as a photoprotection mechanism
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16 (Jeffrey et al., 1997); in spring and summer months, when light intensity increases,
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18 xanthophyll production increases, and diadinoxanthin is transformed into diatoxanthin.
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22 Multi-marker approach and general dynamic of the biofilm

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25 When combining datasets of FAs and pigments together with those on EPS
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27 composition (Fig. 5), samples from every month clearly gathered together, showing a well-
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29 defined temporal evolution. Also, this analysis highlights the association between variables
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31 and samples, which allows following the dynamics of the biofilm in the course of year and to
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33 link EPS composition with microorganisms.
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37 In winter time, the sediment contained high proportions of fucoxanthin and
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39 chlorophyll *c*, which indicated the development of diatoms (Jeffrey et al., 1997); in the
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41 meantime, proportions of most sugars except glucose, particularly galactose and mannose in
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43 both EPS fractions, and scyllo-inositol in colloidal EPS, increased. In spring, the abundance
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45 of diatoms increased, as demonstrated by increase in 16:1 ω 7 and 20:5 ω 3 biomarkers
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47 (Dalsgaard et al., 2003). Also, the increase of alloxanthin proportions together with 18:4 ω 3
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49 FAs mirrored the development of cryptophytes. In late spring, xanthophyll proportions
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51 increased, as a protection against light intensity for diatoms (Jeffrey et al., 1997). Summer
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53 was characterised by high amounts of most saturated FAs, which indicates the accumulation
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55 of degraded organic matter (Balzano et al., 2011; Lü et al., 2010; Sun et al., 1997). This
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57 degradation is emphasised by the presence of chlorophyllide *a* which indicates the presence of
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4 senescing diatoms (Jeffrey et al., 1999); these organisms may then release in the environment
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6 high amounts of intracellular chrysolaminarin, explaining the increase of glucose proportions
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8 in both EPS fraction in summer. Some glucose may also originate from higher photosynthetic
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10 production in late spring and in the beginning of summer, when light intensity, photoperiod
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12 and temperature increase. Finally, autumn is characterised by high levels of markers of
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14 bacterial degradation: pheophytin *a* which results from the degradation of chlorophyll
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16 (Lorenzen, 1967) as well as most branched FAs, which characterise heterotrophic bacteria
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18 (Arts and Wainman, 1999).
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22 Results of analyses on separate datasets showed no correlation between the periods
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24 where microbial assemblages change and those where EPS composition does so (Table 1).
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26 However, our multi-marker analysis suggested links between EPS composition and biological
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28 processes taking place in the sediment, such as photosynthesis, which depend on microbial
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30 assemblages. For instance, the large increase of glucose contribution in summer was linked to
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32 the degradation of senescing diatoms evidenced by chlorophyllide *a*. Finally, it seems that
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34 EPS composition of sediment is influenced at the same time by the succession of microbial
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36 assemblages taking place in tidal flats and directly by environmental conditions.
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43 Conclusion

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46 In this study, association of FA and pigment biomarkers together with monosaccharide
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48 composition of EPS complemented each other to allow a fine characterisation of the biofilm
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50 in the course of year, describing its composition and functioning. Furthermore, as different
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52 monosaccharides may be associated with different ecosystem functions (stabilisation,
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54 recruitment cues for instance; Bellinger et al., 2009; Nelson et al., 2013; Zhou et al., 1998),
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56 this set of variables may indicate diverse roles of biofilms for the ecosystem.
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5

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- 1 Table 1: Results of ANOSIM pairwise post-hoc tests comparing consecutive months (e. g. May with April and June) using 4 sets of markers:
- 2 fatty acids, pigments, colloidal and bound sugars. All 12 samples (pooled from all 3 treatments) from each sampling times were used. Test
- 3 significance: ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Coll: colloidal. Aug: August; Sept: September; Oct: October; Nov:
- 4 November; Jan: January; Feb: February; Mar: March; Apr: April.

	Apr	May	June	July	01Aug	31Aug	Sept	Oct	Nov	Jan12	Fev12	Mar12	Apr12
Fatty acids	***	***	***	***	***	***	**	***	***	ns	Ns	***	
Pigments	ns	ns	ns	ns	*	**	***	ns	ns	***	***	***	
Coll. sugars	*	**	**	*	ns	***	ns	ns	**	ns	**	ns	
Bound sugars	*	ns	ns	ns	**	**	*	*	***	**	Ns	ns	

6 Table 2: Results of ANOSIM pairwise post-hoc tests comparing experimental treatments using 4 sets of markers: fatty acids, pigments, colloidal
7 and bound sugars. All replicates (all samples pooled from all sampling dates) were used for each treatment. Test significance: ns: not significant;
8 *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Treatments were: Ctrl: no tubes; LD: low tube density; HD: high tube density. Tests for colloidal sugars
9 (Coll.sugars) were not performed as global test was not significant.

	Ctrl LD	Ctrl HD	LD HD
Fatty acids	***	***	***
Pigments	*	**	ns
Coll. sugars	-	-	-
Bound sugars	ns	*	*

Table S1: fatty acid percentages in sediment samples (mean over all samples from each treatment and sampling time; \pm standard error).

12:0	0.59	\pm	0.07	16:2w4	0.51	\pm	0.05
13:0	0.05	\pm	0.01	16:2w6	0.19	\pm	0.02
14:0	6.02	\pm	0.17	16:3w4	0.69	\pm	0.09
15:0	3.15	\pm	0.13	16:4w1	0.03	\pm	0.01
16:0	45.38	\pm	1.08	16:4w3	0.09	\pm	0.02
17:0	0.87	\pm	0.03	18:2w6	0.33	\pm	0.04
18:0	6.43	\pm	0.27	18:2w9	0.08	\pm	0.02
19:0	0.07	\pm	0.02	18:3w3	0.06	\pm	0.01
20:0	0.24	\pm	0.02	18:3w6	0.79	\pm	0.13
22:0	0.17	\pm	0.02	18:4w3	0.17	\pm	0.03
24:0	0.13	\pm	0.02	20:2w6	0.84	\pm	0.13
Σ SFA	63.09	\pm	1.43	20:2w9	0.70	\pm	0.12
14:0iso	0.10	\pm	0.01	20:3w6	0.69	\pm	0.09
15:0anteiso	0.69	\pm	0.02	20:4w3	0.09	\pm	0.01
15:0iso	0.83	\pm	0.02	20:4w6	1.02	\pm	0.16
16:0iso	0.29	\pm	0.01	20:5w3	3.19	\pm	0.31
17:0anteiso	0.12	\pm	0.01	22:2w9	0.09	\pm	0.02
17:0iso	0.59	\pm	0.04	22:3	6.02	\pm	0.50
Σ BFA	2.62	\pm	0.08	22:4w6	0.27	\pm	0.05
14:1w3	0.01	\pm	0.00	22:5w3	0.25	\pm	0.04
14:1w5	0.01	\pm	0.00	22:5w6	0.39	\pm	0.07
15:1	0.04	\pm	0.01	22:6w3	0.11	\pm	0.02
16:1w5	0.35	\pm	0.04	Σ PUFA	16.63	\pm	0.45
16:1w7	13.77	\pm	1.21				
16:1w9	0.13	\pm	0.02				
17:1w7	0.02	\pm	0.00				
17:1w9	0.16	\pm	0.02				
18:1w11	0.14	\pm	0.03				
18:1w5	0.08	\pm	0.03				
18:1w7	0.91	\pm	0.09				
18:1w9	1.31	\pm	0.11				
19:1w9	0.04	\pm	0.01				
20:1w11	0.03	\pm	0.01				
20:1w7	0.02	\pm	0.00				
20:1w9	0.02	\pm	0.01				
22:1w11	0.34	\pm	0.06				
22:1w9	0.30	\pm	0.05				
Σ MUFA	17.67	\pm	1.38				

Table S2: pigment percentages in sediment samples (mean over all samples from each treatment and sampling time; \pm standard error).

Chlorophyll <i>a</i>	47.64 \pm 0.37
Chlorophyll <i>b</i>	0.14 \pm 0.04
Chlorophyll <i>c</i>	7.35 \pm 0.17
Chlorophyll <i>a</i> epimer	2.38 \pm 0.08
Chlorophyllide <i>a</i>	4.88 \pm 0.40
Alloxanthin	8.47 \pm 0.25
Diadinoxanthin	4.19 \pm 0.16
Fucoxanthin	23.51 \pm 0.39
Pheophytina	0.96 \pm 0.12
β -Caroten	0.43 \pm 0.03

Table S3: carbohydrate percentages in sediment samples (for each fraction, mean over all samples from each treatment and sampling time; \pm standard error).

	Bound	Colloidal
Fucose	2.62 \pm 0.17	1.67 \pm 0.13
Galactose	5.30 \pm 0.20	5.39 \pm 0.22
Galacturonic acid	0.85 \pm 0.19	0.79 \pm 0.18
Glucose	71.53 \pm 0.87	77.47 \pm 0.82
Glucuronic acid	0.13 \pm 0.02	0.11 \pm 0.03
Mannose	8.29 \pm 0.32	6.89 \pm 0.30
Myo-inositol	1.15 \pm 0.08	0.46 \pm 0.07
Rhamnose	0.76 \pm 0.07	0.94 \pm 0.06
Scyllo-inositol	3.55 \pm 0.36	2.80 \pm 0.23
Xylose	5.81 \pm 0.31	3.49 \pm 0.26

1 **Figures captions**

2 **Fig. 1:** Non-metric Multidimensional Scaling (nMDS) plot of Bray Curtis similarities of the
3 fatty acid (FA) compositions of sediment over the course of the experiment. For each date, all
4 replicates from each treatment are displayed. Data were transformed with Hellinger
5 transformation prior to analysis. The low value of stress indicates that the grouping
6 representation is meaningful: distances between symbols correlate well with dissimilarities
7 between the FA compositions of sediment. Ctrl treatment: circles; LD treatment: squares; HD
8 treatments: triangles. Apr: April; Jun: June; Jul: July; Aug: August; Sept: September; Oct:
9 October; Nov: November; Jan: January; Feb: February; Mar: March.

10 **Fig. 2:** Non-metric Multidimensional Scaling (nMDS) plot of Bray Curtis similarities of the
11 pigment compositions of sediment over the course of the experiment. For each date, all
12 replicates from each treatment are displayed. Data were transformed with Hellinger
13 transformation prior to analysis. The acceptable value of stress indicates that the grouping
14 representation is acceptable: distances between symbols correlate with dissimilarities between
15 the pigment compositions of sediment. Ctrl treatment: circles; LD treatment: squares; HD
16 treatments: triangles. Apr: April; Jun: June; Jul: July; Aug: August; Sept: September; Oct:
17 October; Nov: November; Jan: January; Feb: February; Mar: March.

18 **Fig. 3:** Non-metric Multidimensional Scaling (nMDS) plot of Bray Curtis similarities of the
19 monosaccharide composition in colloidal EPS over the course of the experiment. For each
20 date, all replicates from each treatment are displayed. Data were transformed with Hellinger
21 transformation prior to analysis. The acceptable value of stress indicates that the grouping
22 representation is acceptable: distances between symbols correlate with dissimilarities between
23 the pigment compositions of sediment. Ctrl treatment: circles; LD treatment: squares; HD
24 treatments: triangles. Apr: April; Jun: June; Jul: July; Aug: August; Sept: September; Oct:
25 October; Nov: November; Jan: January; Feb: February; Mar: March.

26 **Fig. 4:** Non-metric Multidimensional Scaling (nMDS) plot of Bray Curtis similarities of the
27 monosaccharide composition in bound EPS over the course of the experiment. For each date,
28 all replicates from each treatment are displayed. Data were transformed with Hellinger
29 transformation prior to analysis. The acceptable value of stress indicates that the grouping
30 representation is acceptable: distances between symbols correlate with dissimilarities between
31 the pigment compositions of sediment. Ctrl treatment: circles; LD treatment: squares; HD
32 treatments: triangles. Apr: April; Jun: June; Jul: July; Aug: August; Sept: September; Oct:
33 October; Nov: November; Jan: January; Feb: February; Mar: March.

34 **Fig. 5:** Results of MFA analysis on sediment samples over the course of the experiment: a.
35 correlation circle between variables used for the analysis; b. confidence ellipses grouping
36 sediment samples from each month. Datasets used for the experiment were fatty acids,
37 pigments, and monosaccharide composition of colloidal and bound EPS. Apr: April; Jun:
38 June; Jul: July; Aug: August; Sept: September; Oct: October; Nov: November; Jan: January;
39 Feb: February; Mar: March. Chla, Chlb, Chlc: chlorophylls a, b and c, respectively. C:
40 Colloidal EPS. B: Bound EPS. AGlu: Glucuronic acid. AGal: galacturonic acid. Fuc: Fucose.
41 Gal: Galactose. Glu: Glucose. Man: Mannose. Myo: Myo-inositol. Rha: Rhamnose. Scyl:
42 Scyllo-inositol. Xyl: Xylose.

43

Figure(s)
Fig. 1

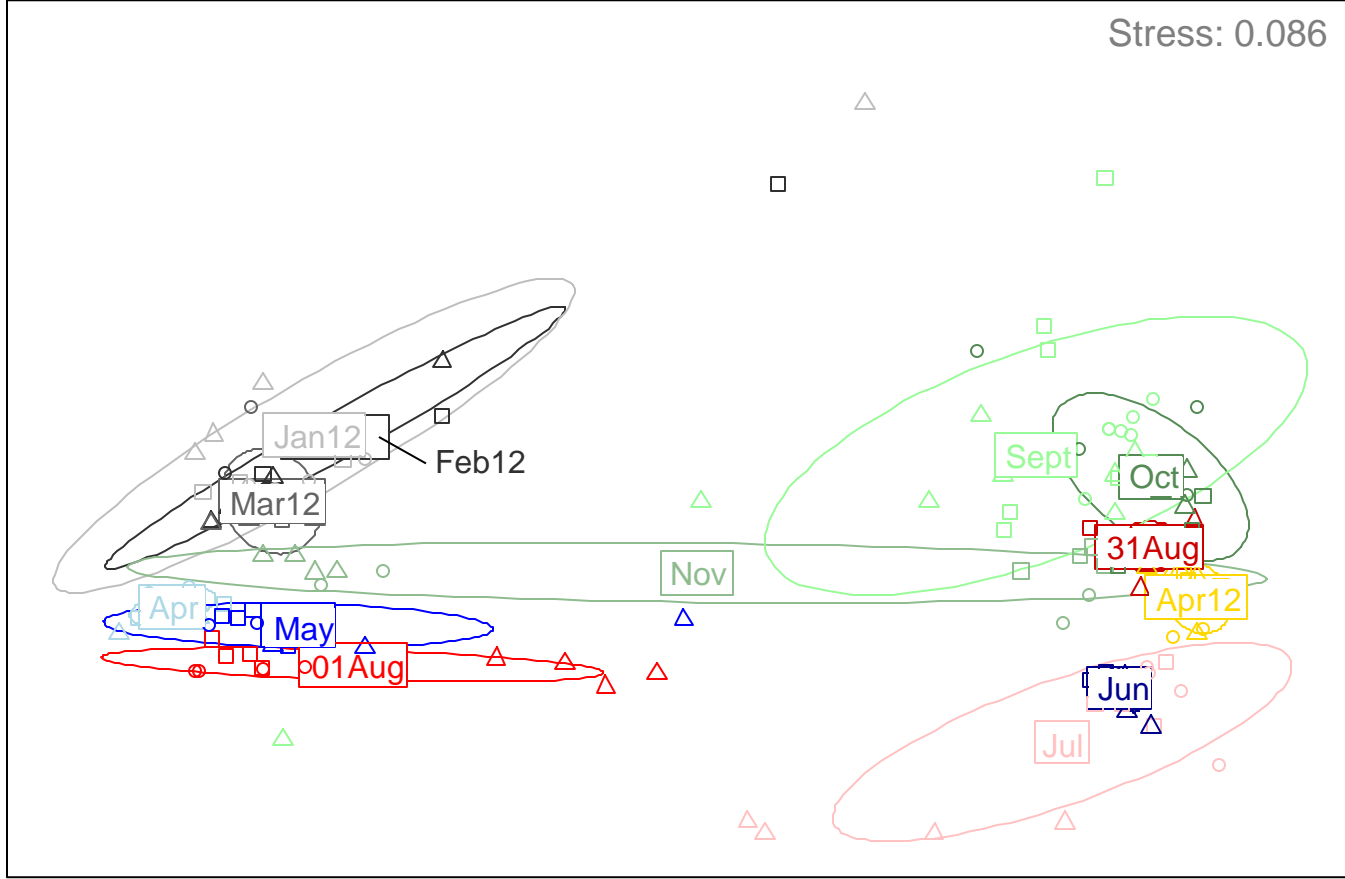


Fig. 2

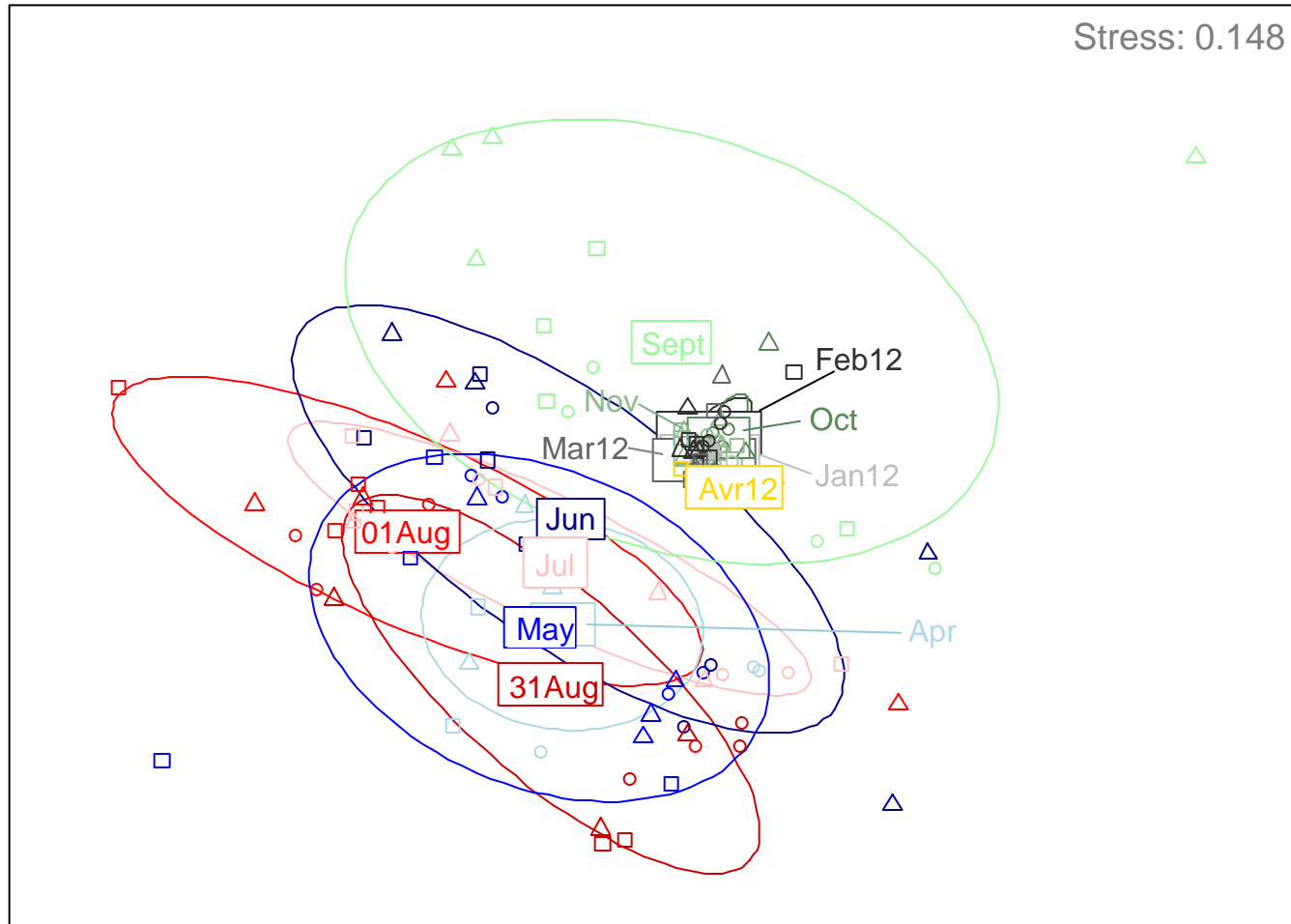


Fig. 3

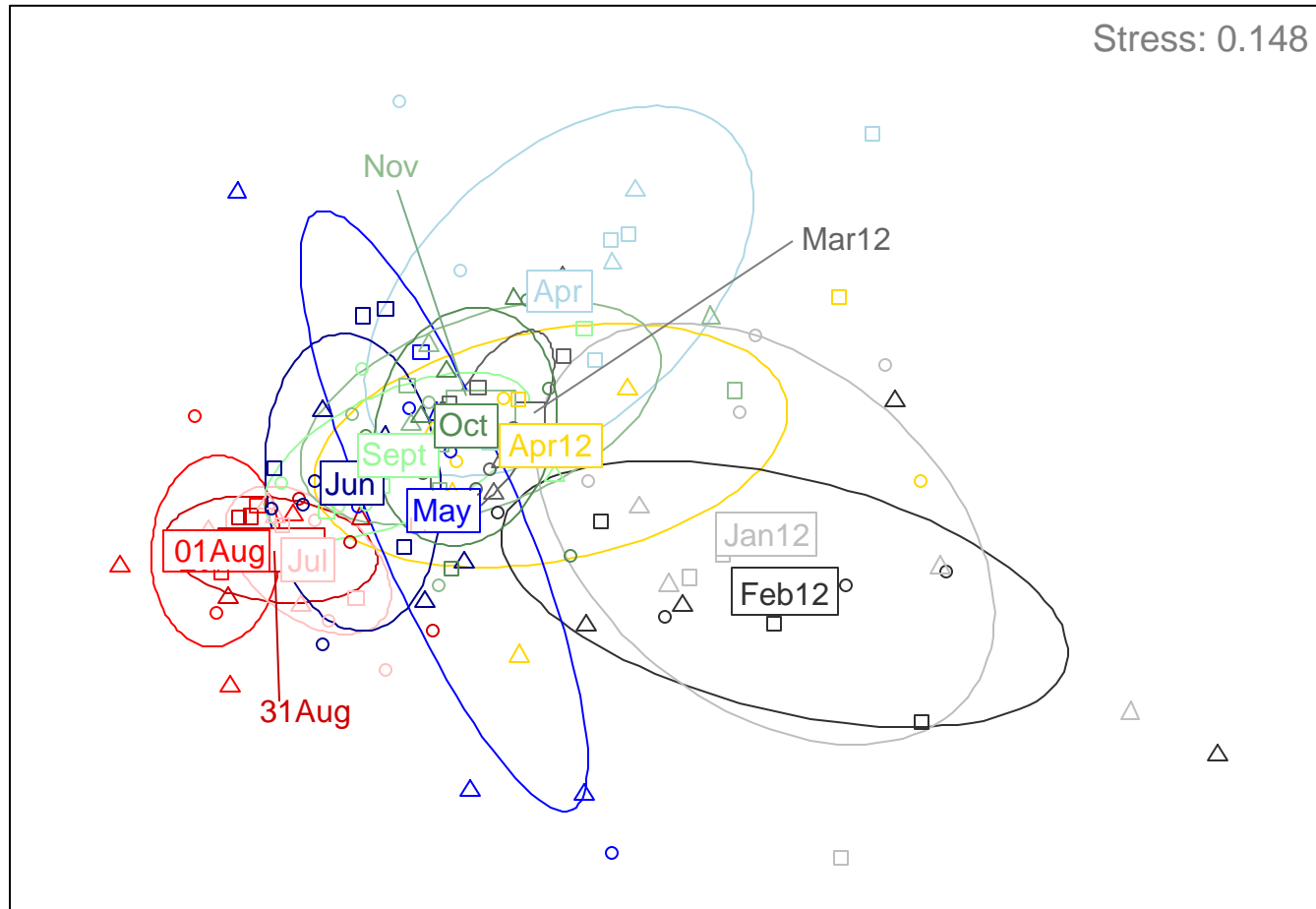


Fig. 4

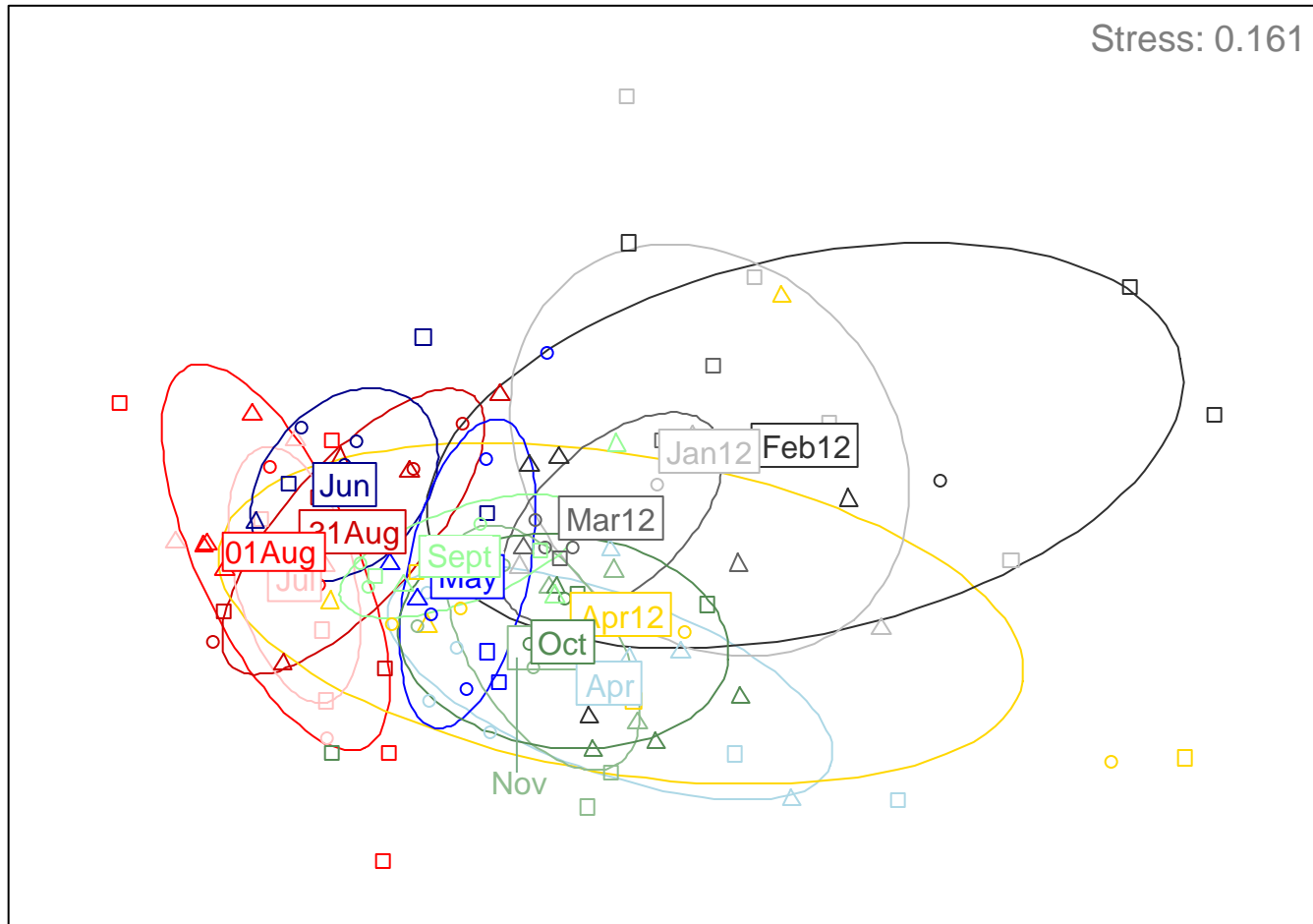
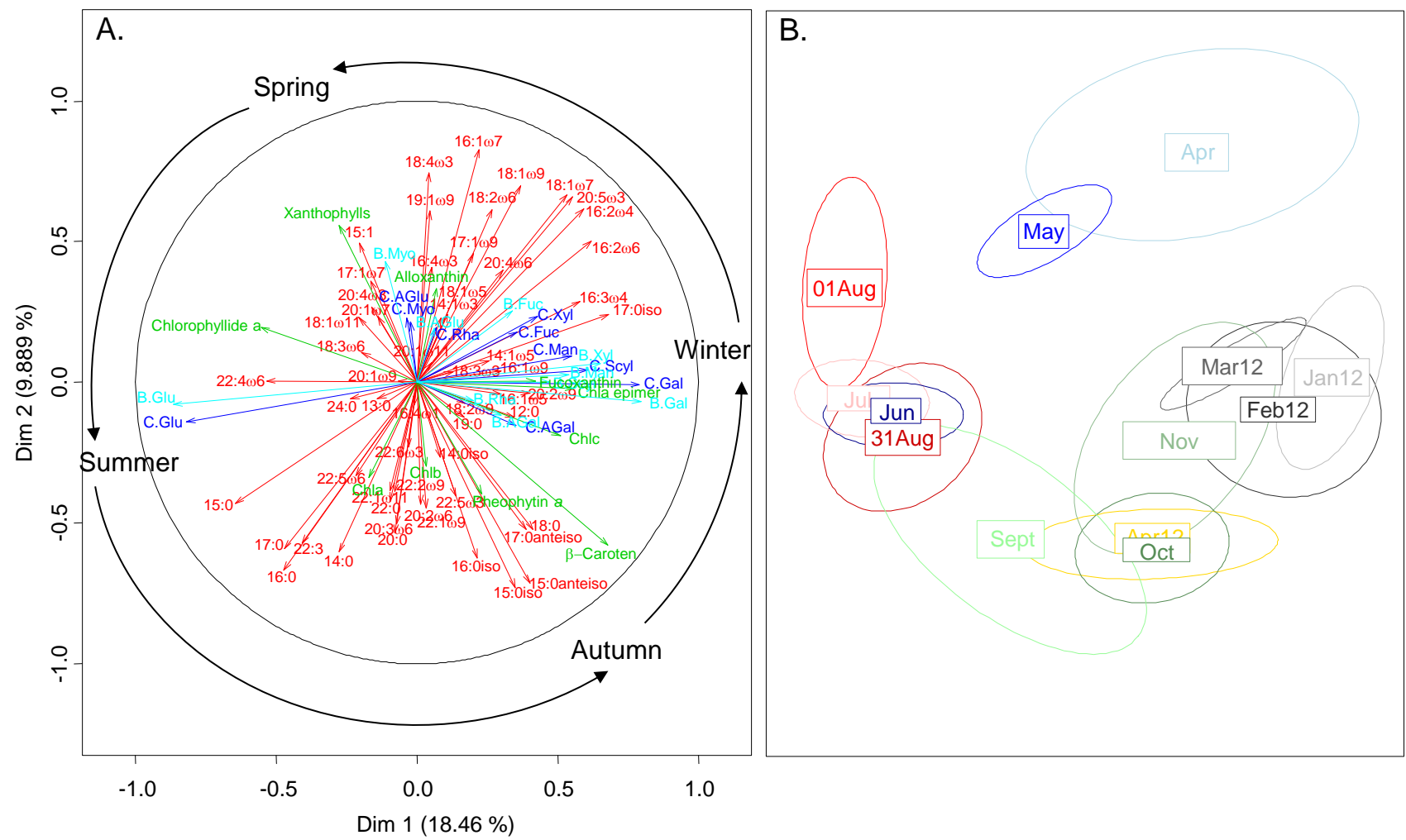


Fig. 5



Supplementary figures: captions

Fig. S1: evolution of total fatty acid (FA) concentrations in sediment over the course of the experiment. The x axis displays the number of days from the beginning of the experiment (March 21st, 2011). Treatments were : no tubes (Ctrl, grey circles); low tube density (LD, empty square) and high tube density (HD, black triangles).

Fig. S2: evolution of total pigment concentrations in sediment over the course of the experiment. The x axis displays the number of day from the beginning of the experiment (March 21st, 2011). Treatments were : no tubes (Ctrl, grey circles); low tube density (LD, empty square) and high tube density (HD, black triangles).

Fig. S3: evolution of total EPS concentrations: a. carbohydrates and b. proteins in sediment over the course of the experiment. The x axis displays the number of day from the beginning of the experiment (March 21st, 2011). Treatments were : no tubes (Ctrl, grey circles); low tube density (LD, empty square) and high tube density (HD, black triangles).

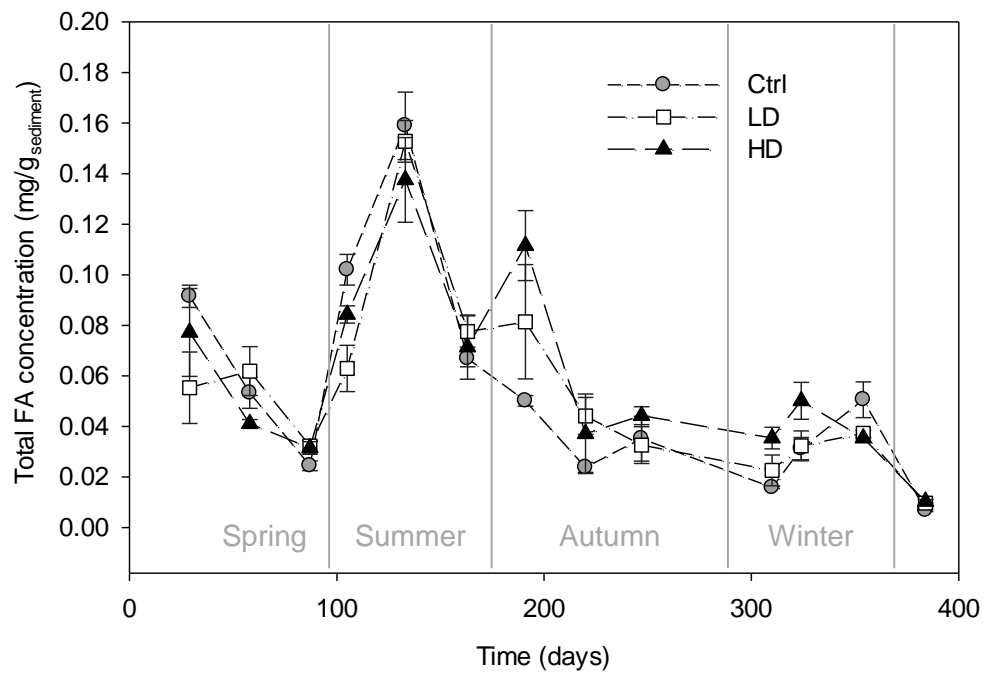


Fig. S2

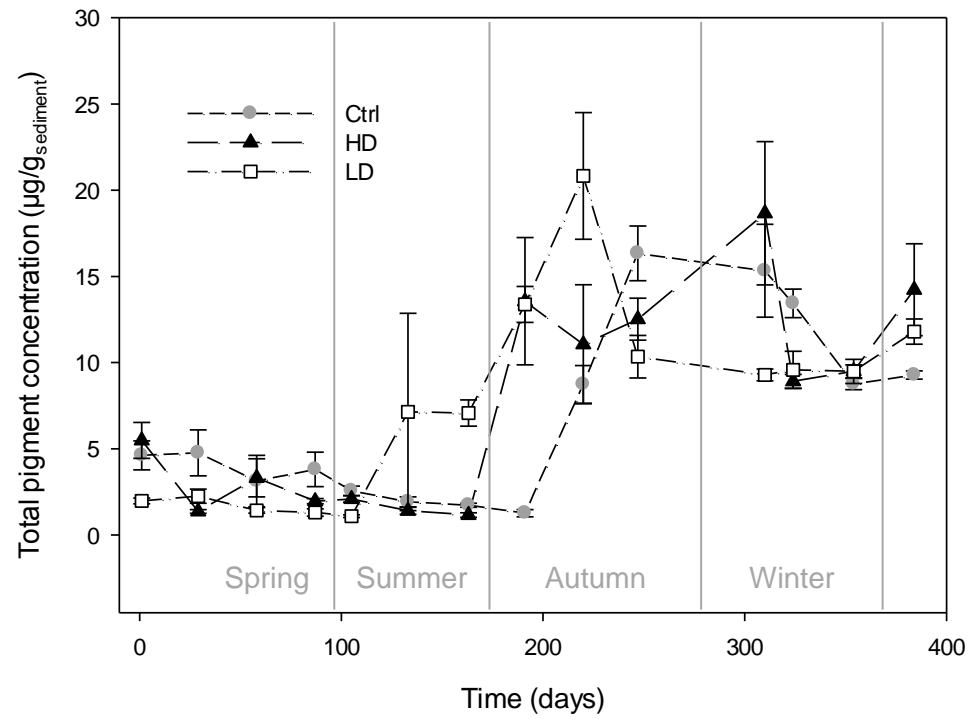


Fig. S3

