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Running title: Prokaryote drivers in mudflats

1 Highlights

- Strong stratification in two horizons of microbial densities and activities in the first 10 cm of the sediment.
- A gradual transition could correspond to an environmental ecocline rather than an ecotone.
- Bottom-up-control of the prokaryotic community revealed by the variation partitioning analysis.

2 Abstract

Intertidal mudflats are rich and fluctuating systems. The upper 20 cm support a high diversity and density of microorganisms that ensure diversified roles. The depth profiles of microbial abundances and activities were measured in an intertidal mudflat (Marennes-Oléron Bay, SW France) at centimeter-scale resolution (0-10 cm below the sediment surface). The aim of the study was to detect microbial stratification patterns within the sediments and how this stratification is shaped by environmental drivers. Two sampling dates, *i.e.* one in summer and another in winter, were compared. The highest activities of the microbial communities were observed in July in the surface layers (0-1 cm), with a strong decrease of activities with depth. In contrast, in February, low microbial bulk activities were recorded throughout the sediment. In general, prokaryotic abundances and activities were significantly correlated. Variation partitioning analysis suggested a low impact of predation and a mainly bottom-up-controlled prokaryotic community. Hence, in the top layer from the surface to 1–3.5 cm depth, microbial communities were mainly affected by physicochemical variables (*i.e.*, salinity, phosphate and silicate concentrations). Below this zone and at least to 10 cm depth, environmental variables were more stable and prokaryotic activities were low. The transition zone between both layers probably represents a rather smooth gradient (environmental ecocline). The results of our study provide a better understanding of the complex interactions between micro-organisms and their environment in a fluctuating ecosystem such as an intertidal mudflat.

Keywords: intertidal mudflat, sediment depth, microbial communities, benthic ecology

3 Introduction

In temperate zones, intertidal mudflats are among the most productive coastal ecosystems due to the development of an active microphytobenthic biofilm at the surface of the sediment (Admiraal, 1984; Underwood and Kromkamp, 1999). Several factors drive the high productivity levels such as incident light and large nitrogen-rich inputs from the continent (Underwood and Kromkamp, 1999), in these complex ecosystems. The knowledge about the relationship between the microphytobenthos and the activity of prokaryotic communities, although recognized as of paramount importance for determining the productivity of these ecosystems (Agogué et al., 2014; Decho, 2000; McKew et al., 2013; Orvain et al., 2014a), is still largely insufficient (Van Colen et al., 2014). Marine coastal sediments harbor among the most diverse and abundant prokaryotic communities (Whitman et al., 1998; Zinger et al., 2011). The abundances and activities of these microbial communities seem to vary along a vertical gradient at a restricted vertical scale (*e.g.*, < 20 cm) under the influence of the 1) organic matter composition and quality and electron acceptor availability (Kristensen, 2000), 2) physical properties of the sediments, 3) bioturbation and bioirrigation activities 4) bottom-up and top-down trophic controls, and 5) climatic conditions.

The dominant source of carbon for heterotrophic microorganisms in temperate intertidal mudflats is derived from microphytobenthic activities (*i.e.*, photosynthesis and exopolymeric substance production) (Underwood and Kromkamp, 1999). This organic matter production is primarily ensured by epipelic (*i.e.*, motile free-living) diatoms and quickly transferred to other biological compartments (Middelburg et al., 2000). The microphytobenthic biofilm has such a relevant effect on prokaryotic communities at low tide that it drastically modifies the remineralization and fluxes of inorganic nutrients across the sediment surface (Middelburg et al., 2000).

In muddy fine-grained low-permeable sediments, where advection fluxes are almost absent, physical transport of solutes is mainly driven by molecular diffusion within the interstitial water. The top sediment layers show a strong consumption of oxygen by organotrophic microorganisms and by reoxidation of reduced compounds such as Fe^{2+}, Mn^{2+}, H_2S (Soetaert et al., 1996). Hence, oxygen does not diffuse below the first few millimeters in mudflats where deeper sediment are most often anoxic (Bertics and Ziebis, 2010). Other inorganic electron acceptors, including the nutrient nitrate, can thus be used deeper in the sediment by dissimilatory processes (*e.g.*, denitrification) for anaerobic mineralization. Hence, microbial communities may exhibit vertical patterns in the nature and rate of their activity in response to changing biogeochemical conditions, implicating different prokaryotic assemblage.

Moreover, infauna activity plays a crucial role in the modulation of microbial activity in sediments by disturbing the vertical gradients of oxygen, organic matter and inorganic nutrients (Bertics and Ziebis, 2009; Gilbertson et al., 2012; Jones et al., 1996). As an example, prokaryotic activity has been shown to be increased by both bioirrigation and bioturbation activities in a coastal lagoon of the Santa Catalina Island (CA, USA) (Bertics and Ziebis, 2009).

Furthermore, prokaryotes may strongly vary under trophic controls. The impact of the availability of resources (*e.g.*, organic matter and/or inorganic nutrients) is defined as the bottom-up control of the microbial communities (Fuhrman and Hagström, 2008), and may strongly change at both spatial and temporal scales. On the other hand, top-down control is described as grazing pressure primarily carried out by meiofauna or viruses (*i.e.*, prokaryotic cell lysis) in intertidal mudflats. Among the few studies focusing on the balance of bottom-up/top-down control in mudflats, the role of top-down control by meiofauna seems to be significant and could be more important than bottom-up control (Fabiano and Danovaro, 1998), although a local study indicated that grazing pressure did not represent a crucial control of bacterial community (Pascal et al., 2009). In a microcosm study, De Mesel et al. (2004)

highlighted that both trophic controls have to be considered as bacterial community structure is a function of substrate but the relative abundance of each taxa is influenced by the grazing activities of bacterivorous nematods.

Finally, in intertidal zones and especially in macrotidal systems, the alternation of emersion and immersion produces drastically fluctuating conditions, particularly during low tide at the sediment surface. For example, temperature, a key factor impacting prokaryotic metabolism in coastal sediments (Hubas et al., 2007), can fluctuate significantly within 6 hours of a low tide (until 16°C of amplitude measured at the sediment surface in the Marennes Oléron mudflat, France, Orvain et al. (2014a)). Moreover, in these shallow ecosystems, other climatic conditions such as wind or waves can strongly disturb the global (*i.e.*, biotic and abiotic) vertical zonation of the sediment (Dupuy et al., 2014).

The aims of this study were 1) to describe stratification patterns of the activities and densities of prokaryotic communities in coastal mudflats at cm-scale spatial resolution and 2) to statistically disentangle the relative contributions of environmental variables and meiofauna abundances in the different depth layers as possible drivers for these prokaryotic activities and densities. This work was focused on an intertidal mudflat in Marennes-Oléron Bay (SW France) sampled twice at low tide, during representative summer and winter conditions, respectively in assessed how the patterns of prokaryotic densities and activities varied with depth and to identify the impact of physicochemical variables and potential grazing pressure on the stratification observed.

4 Materials and Methods

4.1 Study site and sampling

Sediment cores were sampled in Marennes-Oléron Bay on the Atlantic French coast (1 km from the shore) (N 45° 54′ 53″; W 01° 05′ 23″). The intertidal mudflat is characterized by the presence of parallel ridges and runnels and sampling was performed on ridges at low tide. Two sampling dates were compared at a similar tidal range (5.5 ± 0.2 m): 1) on July 5, 2012, high temperature and incident irradiance and 2) on February 11, 2013, low temperature and incident irradiance.

On each sampling date, triplicate 15-cm diameter cores were sliced *in situ* into five layers using a piston inserted below the core from 0 to 10 cm below the sediment surface (bsf) (D1 = 0-0.5 cm; D2 = 0.5-1 cm; D3 = 1-2 cm; D4 = 2-5 cm and D5 = 5-10 cm). Samples were homogenized and subdivided using 50-ml sterile syringes with cut-off tips for further analysis (storage conditions differed according to the variable, see Supp info Table S1). Triplicate cores 12-cm in diameter were simultaneously recovered for the determination of pore-water nutrient concentrations. These cores were pre-drilled vertically at 0.5 cm resolution, and pore water was collected at 0.5, 1, 1.5, 3.5 and 7.5 cm bsf, using the Rhizons® (Rhizosphere Research Products Netherlands) method (Seeberg-Elverfeldt et al., 2005). The Rhizons were inserted horizontally into the sediment core during 20 minutes to collect enough pore-water volume for subsequent analysis.

4.2 Physical and chemical analysis

Incident irradiance and temperature at the surface of the sediment were assessed *in situ* every 30 seconds with a universal light-meter and data logger (ULM-500, Walz Effeltrich, Germany) equipped with a plane light/temperature sensor (accessory of the ULM-500) and a plane cosine quantum sensor (Li-COR, USA). Depth temperature profiles were measured every

30 seconds during all the sampling period with five 3.1-cm length Hobo sensors (Hobo Pro V2, USA) fixed on a homemade stick that was vertically pushed into the sediment to position the sensors at 5 different depths (0.5 cm, 1 cm, 2 cm, 5 cm and 10 cm bsf).

At the laboratory, pore-water pH and salinity (using the Practical Salinity Scale) were measured in the supernatant after centrifugation (15 min, 3,000 $\times g$ at 8 °C) with a pH probe (Eutech Instruments PC150, USA) and a conductivity meter (Cond 3110, TetraCon 325, WTW, Germany), respectively. Sediment density and porosity were evaluated by weighing 50 ml of fresh sediment before and after drying (48 h at 60 °C). Porosity was calculated as the ratio of the volume of water divided by the total volume of sediment. After removal of salts and organic matter, the mean grain size of the sediment was measured by a laser granulometer (Mastersizer 2000, Malvern Instruments, U.K.) and evaluated using the GRADISTAT program (Blott and Pye, 2001) according to the Folk and Ward theory (Folk and Ward, 1957).

Total organic carbon (TOC) and total nitrogen (TN) contents were measured on lyophilized samples by oxic combustion at 950°C (Strickland and Parsons, 1972) using a CHN elemental analyzer (Thermo Fisher Flash EA 1112, Waltham, MA, USA). Samples for TOC were decarbonated (in hydrochloric acid, HCl 1N) prior to combustion to remove the inorganic carbon. Because the decarbonation could biased the TN content analysis, subsamples were ran before and after decarbonation to validate the TN measurement.

Two exopolymeric (EPS) fractions (colloidal and bound) were extracted in two steps: colloidal EPS were extracted using fresh sediment mixed with an equal volume of artificial seawater, then bound EPS were extracted using the residual sediment mixed with Dowex resin (Takahashi et al., 2009). Before quantification of EPS-proteins and -carbohydrates, each extract was vacuum-evaporated over 6h (Maxi Dry plus, Heto, Denmark). Colloidal and bound EPS-protein concentrations were determined using the bicinchoninic acid assay (Smith et al., 1985). Colloidal and bound EPS-carbohydrate concentrations were determined according to the

167 phenol-sulfuric acid method (Dubois et al., 1956). The four resulting fractions colloidal EPS-
168 proteins, bound EPS-proteins, colloidal EPS-carbohydrates and bound EPS-carbohydrates were
169 expressed in µg g^{-1} sed DW. Colloidal EPS correspond to the sum of colloidal EPS-proteins
170 and colloidal EPS-carbohydrates. Bound EPS correspond to the sum of bound EPS-proteins
171 and bound EPS-carbohydrates. Colloidal EPS and bound EPS were used for the calculation of
172 the ratio colloidal EPS / bound EPS. EPS-carbohydrates correspond to the sum of colloidal
173 EPS-carbohydrates and bound EPS-carbohydrates. EPS-proteins correspond to the sum of
174 colloidal EPS-proteins, bound EPS-proteins. EPS-carbohydrates and EPS-proteins were used
175 for the calculation of the ratio EPS-carbohydrates/EPS-protein.

176 Total protein content was determined in sediment (stored at -20 °C) after extraction (30
177 min, in the dark, +4 °C in 0.2-µm-filtered seawater) using Lowry Peterson's modification assay
178 (Sigma-Aldrich). Ammonium (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-), phosphate (PO_4^{3-}), and
179 silicate ($Si(OH)_4$) concentrations were determined using an autoanalyzer (Seal Analytical,
180 GmbH Nordertedt, Germany) equipped with an XY-2 sampler according to Aminot and
181 Kérouel (2007).

182 *4.3 Biotic parameters*

183 Chlorophyll *a*, used as a proxy of algal biomass, was assessed by fluorimetry (640 nm,
184 Turner TD 700, Turner Designs, USA) after extraction with 90% acetone. Chlorophyll *a*
185 concentrations were expressed as µg g^{-1} sediment dry weight (DW) according to Lorenzen
186 (1966). Prokaryotic abundance was evaluated by flow cytometry after a cell extraction
187 procedure described by Lavergne et al. (2014).

188 Analyses of the two potential extracellular enzymatic activities, β-glucosidase and
189 aminopeptidase, were determined by spectrofluorimetry (Boetius, 1995) (SAFAS Scientific
190 Instruments, Monaco) [excitation/emission = β-glucosidase activity: 365 nm/460 nm; and

aminopeptidase: 340 nm/410 nm]. For β-glucosidase activity, slurry sediment samples were incubated in triplicate using 4-Methylumbelliferyl β-D-glucopyranoside (500 µmol L^{-1} final conc.) as a substrate at three different incubation times: 15, 45, and 75 min. For aminopeptidase activity, slurry sediment samples were incubated in triplicate with L-leucine β-naphthylamide hydrochloride (300 µmol L^{-1}, final conc.) as a substrate at three different incubation times: 10, 30, and 60 min. Final concentrations of 4-Methylumbelliferyl β-D-glucopyranoside and L-leucine β-naphthylamide hydrochloride were determined previously to represent saturation levels and maximum yield velocities (V_{max}) (Boetius and Lochte, 1996).

Incorporation of [methyl-^{3}H] thymidine into DNA was measured as a proxy of benthic bacterial production (Garet and Moriarty, 1996; Pascal et al., 2009). Briefly, 30 µl of fresh sediment slurry (vol/vol; 0.2-µm-filtered seawater) was incubated with ^{3}H-thymidine 0.74 × 10^6 Bq for 1 h at *in situ* temperature (22°C in July and 12°C in February). Blank controls were stopped just after the addition of labelled ^{3}H-thymidine with 8 ml of cold 80% ethanol. After incubation, samples were stopped with 8 ml of cold ethanol (80%). After two washes with 80% cold ethanol by mixing and centrifugation (15 min, 4 500 g, +4°C), slurries were transferred with 2 mL of ice-cold TCA (5%, trichloroacetic acid) onto a polycarbonate filter (Nuclepore 0.2 µm, 25 mm, Millipore, NJ, USA). Subsequently, the filters were washed four times with 5% ice-cold TCA. Afterwards, the filters were transferred into scintillation vials containing 2 ml 0.5N chlorhydric acid and incubated 16 h at +95°C (Garet and Moriarty, 1996). Supernatant (0.5 mL) was transferred in a new scintillation vial with 5 mL of scintillation fluid (Ultima Gold, Perkin-Elmer, MA, USA). The amount of radioactivity in each vial was measured using a scintillation counter (Perkin-Elmer, USA). Benthic bacterial production was finally expressed as pmol ^{3}H Thy g^{-1} sed DW h^{-1} using a conversion factor 4.51 × 10^{-13} (Ci dpm^{-1}) evaluated experimentally to account for counter efficiency.

For meiofaunal assemblage determination, samples from each depth (60 mL) were stored directly after sampling at room temperature in absolute ethanol, sieved through 50 µm before staining with rose Bengal and observation under stereo microscope (Zeiss). Foraminifera were counted in all the sediment samples, and for other meiofauna organisms (*i.e.,* juvenile gastropods, copepods, ostracods, nematods, foraminifera, and juvenile bivalves), samples were diluted prior to counting. Abundances were expressed as individuals (ind.) per cm^3. Additionally, six 20-cm diameter PVC cores were harvested at each date and sieved through 1 mm. The macrofauna was collected and stored in ethanol 60% for further identification. In the current study, only the data of the abundance of the macrozoobenthic grazer, *Peringia ulvae* (Pennant, 1777) are presented. The mean abundance of the six cores is expressed in ind m^{-2}.

4.4 Statistical analyses

All statistical analyses were performed with R software (R Core Team, 2013). In this study, the results are presented as the means ± standard error (SE) because the SE evaluates the mean estimation imprecision. To evaluate the effect of temperature on thymidine incorporation rate, Q_{10} values were calculated at each sampling depth (Lomas et al., 2002) using the Equation 1.

Equation 1: $Q_{10} = (R2/R1)^{\left(10/t_2 - t_1\right)}$

Where *R2* and *R1* are the thymidine incorporation rates and t_2 and t_1 are the incubation temperatures in July and February, respectively. This factor indicating the increase of a process rate with 10°C increase of temperature is more powerful when calculated with large dataset and/or used with regression data (Hubas et al., 2007; Lomas et al., 2002). In the current study, as the replicates are independent between the two sampling dates, Q_{10} factor was calculated for

all the possible combinations ($n=3^3$). Then, a student test for one sample was used to evaluate whether the Q_{10} values were significantly different from 1.

Pearson tests were used to test whether the distribution of two variables was similar. The significant variation of environmental and prokaryotic variables among sediment depths and sampling dates was evaluated by two-way ANOVA (using sampling date - 2 levels - and depths - 5 levels - as factors) followed by multiple comparison tests (Tukey HSD test) and variance homogeneity and residuals normality were tested. For the two variables "chlorophyll a" and "Q_{10} of thymidine incorporation", the ANOVA assumptions were violated, the variables were ln-transformed and two-way ANOVA followed by Tukey HSD test was performed. For the two variables "EPS-carbohydrates/EPS-protein" and "colloidal EPS/ bound EPS", the ANOVA assumptions were violated and transformation was not possible, non-parametric Friedman test was thus run followed by the Nemenyi post-hoc test for multiple joint samples (Nemenyi, 1963; Sachs, 1997) using the "PMCMR" package (Pohlert, 2014).

Multivariate principal component analysis (PCA) was performed for July sampling and February sampling separately with 8 environmental variables using the "FactoMineR" package (Husson et al., 2013). Then, in order to define sediment horizons using the basis of each PCA obtained, a hierarchical clustering analysis was applied using the HCPC function of the "FactoMineR" package (Husson et al., 2013).

Finally, in order to disentangle the impacts of the environmental variables and meiofaunal group abundance, both taken individually as well as shared, on the distribution of prokaryotic density and activities, variation partitioning was performed (Borcard et al., 1992; Ramette, 2007; Volis et al., 2011) using the varpart function of the "vegan" packages (Oksanen et al., 2013)). First, one response table and three explanatory tables were built and composed as follows. The response table corresponds to the "prokaryotic" table (P table) containing prokaryotic abundance (PA), thymidine incorporation (Thy.inc), aminopeptidase activity

(AMA), and β-glucosidase activity (BGA) and was standardized to unit variance. The explanatory "meiofauna" table (M table) containing abundances of juvenile gastropods, copepods, ostracods, nematods, foraminifera, and juvenile bivalves was log10(x + 1) transformed to normalize the distribution. And the explanatory table of the "environmental variables" (E table) (standardized) contains temperature, salinity, pH, the ratio DIN:PO_4^{3-}, the ratio TOC:TN, total protein content, porosity, EPS-carbohydrates/EPS-protein and colloidal EPS/bound EPS.

Using forward selection procedure (Legendre and Legrendre, 1998) with the function forward.sel in the package 'packfor' (Dray et al., 2013), we selected the variables that influenced the most the response table (Ramette and Tiedje, 2007). The final explanatory tables was thus composed as follows: the table E containing phosphate and silicate concentrations as well as salinity and the table M containing abundance of juvenile gasteropods. The variation partitioning evaluates diverse components of variation of a set of response variables: 1) the pure effect of each individual explanatory table without the effect of the other explanatory table; 2) the redundancy of the two explanatory tables which is the part of the variance explained by both explanatory tables; and 3) the residual effects unexplained by the chosen variables (Borcard et al., 1992; Volis et al., 2011). In this set of data, it is expected that the distribution of abundances and activities of prokaryotes (P table) responds linearly to the explanatory variables, thus we used the linear-based PCA and redundancy analysis (RDA) for the analysis. The total variance to be explained was evaluated by a PCA with the abundances and activities of prokaryotes (P table). RDA was used to assess the amount of variation of the P table explained by the two explanatory variables (as constraining variables). Using partial RDA (pRDA), the effect of a set of variable (an explanatory table) could be removed from the analysis if selected as a covariable. This is an important issue of this multivariate analysis that, in the present case, evaluates for example the effect of meiofauna on prokaryotic variables without the effect of the

environmental variables. These environmental variables such as salinity could indeed be important factors for both prokaryotic and meiofaunal communities and the partition of these effects allows to quantify the pure effect of the meiofauna without the shared variation with environmental variables. Finally, the significance of each ordination was tested by an ANOVA like permutation test using 9999 permutations (Volis et al., 2011).

5 Results

5.1 Environmental conditions and variation of physicochemical variables

The air temperature and incident irradiance at the surface of the mudflat were 28 ± 0.9 °C and 1800 ± 156 µmol photons m^{-2} s^{-1} and 10.5 ± 1.1 °C and 611 ± 292 µmol photons m^{-2} s^{-1} during the samplings in July and in February, respectively (Supp info Fig. S1 and Table S2). The sediment was predominantly silt-clay (mean of 91.2%), with an average grain size of 11.17 ± 0.34 µm and a porosity of 0.73 ± 0.01.

Two-way ANOVA reveals that all the physicochemical variables (presented in the Table 1) were significantly different between the two sampling dates ($p < 0.05$) except the Colloidal EPS / bound EPS ratio. Significant variations with sediment depth are highlighted by Tukey's post hoc test (Table 1, see Supp info Fig. S2 to Fig. S4 for detailed profiles).

Then, two principal component analysis (PCA) were performed using 8 variables (*i.e.*, presented in the Table 1 except grain size, porosity and algal biomass) aiming at describing the interactions within the physicochemical variables for each sampling date and a hierarchical clustering analysis based on the ordinations obtained was used to group the samples. In July, the two first dimensions of the PCA together explained 66.68 % of the observed variability in the dataset (Figure 1a). The first dimension was mostly characterized by TOC:TN, pH, salinity and temperature and differentiated the samples in two groups from 0 to 1 cm bsf on one hand

and the samples from 1 to 10 cm bsf on the other hand (Figure 1c). In February, the two first dimensions of the PCA together explained 68.94 % of the observed variability in the dataset (Figure 1b). The first dimension was mostly characterized by TOC:TN, pH and temperature and differentiated the samples in two groups from 0 to 2 cm bsf on one hand and the samples from 2 to 10 cm bsf on the other hand (Figure 1d).

In both cases, DIN:PO_4^{3-} and EPS-carbohydrates/EPS-proteins ratios have information represented in both dimensions of the ordinations.

317 **Table 1.** Average of each environmental variables and algal biomass (± SE) along sediment depths. Two-way ANOVA reveals always significant d

318 letters in bold font indicate Tukey's post hoc test for each sampling date.

	Grain size[1]		**Porosity**			**Salinity**			**pH**			**Temperature**			**TOC:TN**[2]			**DIN:PO_4^{3-}**			**car**
	µm											°C									
	Mean	*SE*	Mean	*SE*		Mean	*SE*		Mean	*SE*		Mean	*SE*		Mean	*SE*		Mean	*SE*		Mea
July 5th, 2012																					
D1 (0-0.5 cm)	9.1	*0.3*	0.78	*0.02*	**a**	45.4	*2.3*	**a**	7.5	*0.1*	**a**	22.2	*2.9*	**a**	6.06	*0.03*	**a**	9.10	*3.86*	**a**	2,
D2 (0.5-1 cm)	8.7	*0.5*	0.72	*0.01*	**ab**	37.5	*0.2*	**b**	7.0	*0.0*	**b**	22.2	*0.9*	**a**	6.27	*0.03*	**a**	54.65	*21.12*	**ab**	4,
D3 (1-2 cm)	9.3	*0.1*	0.69	*0.03*	**ab**	34.3	*0.1*	**b**	6.7	*0.1*	**b**	21.9	*0.6*	**a**	6.45	*0.03*	**a**	21.88	*1.09*	**b**	1,
D4 (2-5 cm)	10.7	*0.2*	0.69	*0.00*	**b**	33.5	*0.8*	**b**	6.6	*0.0*	**c**	21.5	*0.3*	**ab**	6.62	*0.09*	**a**	175.10	*100.58*	**ab**	1,
D5 (5-10 cm)	11.0	*0.4*	0.68	*0.02*	**b**	33.4	*0.5*	**b**	6.9	*0.1*	**b**	20.8	*0.2*	**b**	6.59	*0.04*	**a**	241.58	*79.25*	**ab**	0,
February 11th, 2013																					
D1 (0-0.5 cm)	14.4	*NA*	0.77	*0.03*	**a**	24.3	*0.2*	**ab**	7.9	*0.0*	**a**	9.5	*2.1*	**a**	6.75	*0.17*	**a**	57.09	*14.26*	**a**	0,
D2 (0.5-1 cm)	13.2	*NA*	0.75	*0.00*	**ab**	23.5	*0.8*	**a**	7.2	*0.1*	**b**	8.8	*1.2*	**ab**	7.20	*0.08*	**a**	18.66	*7.84*	**abc**	0,
D3 (1-2 cm)	10.5	*NA*	0.79	*0.03*	**ab**	23.6	*1.0*	**ab**	7.2	*0.2*	**b**	9.2	*0.5*	**ab**	7.35	*0.13*	**b**	6.87	*2.06*	**ab**	0,
D4 (2-5 cm)	11.9	*NA*	0.71	*0.02*	**b**	26.3	*1.8*	**ab**	7.0	*0.1*	**b**	8.8	*0.7*	**ab**	7.47	*0.13*	**b**	11.52	*1.74*	**bc**	0,
D5 (5-10 cm)	13.0	*NA*	0.69	*0.01*	**b**	29.5	*1.7*	**b**	7.0	*0.1*	**b**	8.4	*0.2*	**b**	8.00	*0.16*	**b**	19.54	*6.14*	**c**	0,

[1]Note that triplicates were not available for grain size for February sampling (NA: not available)

[2]TOC:TN : ratio of total organic carbon (TOC) to total nitrogen (TN). TOC and TN are in µg g^{-1} sed DW

[3] EPS-carbohydrates/EPS-protein and colloidal EPS/bound EPS are ratios without unit. Colloidal EPS-proteins, bound EPS-proteins, colloidal EPS-carbohydrat

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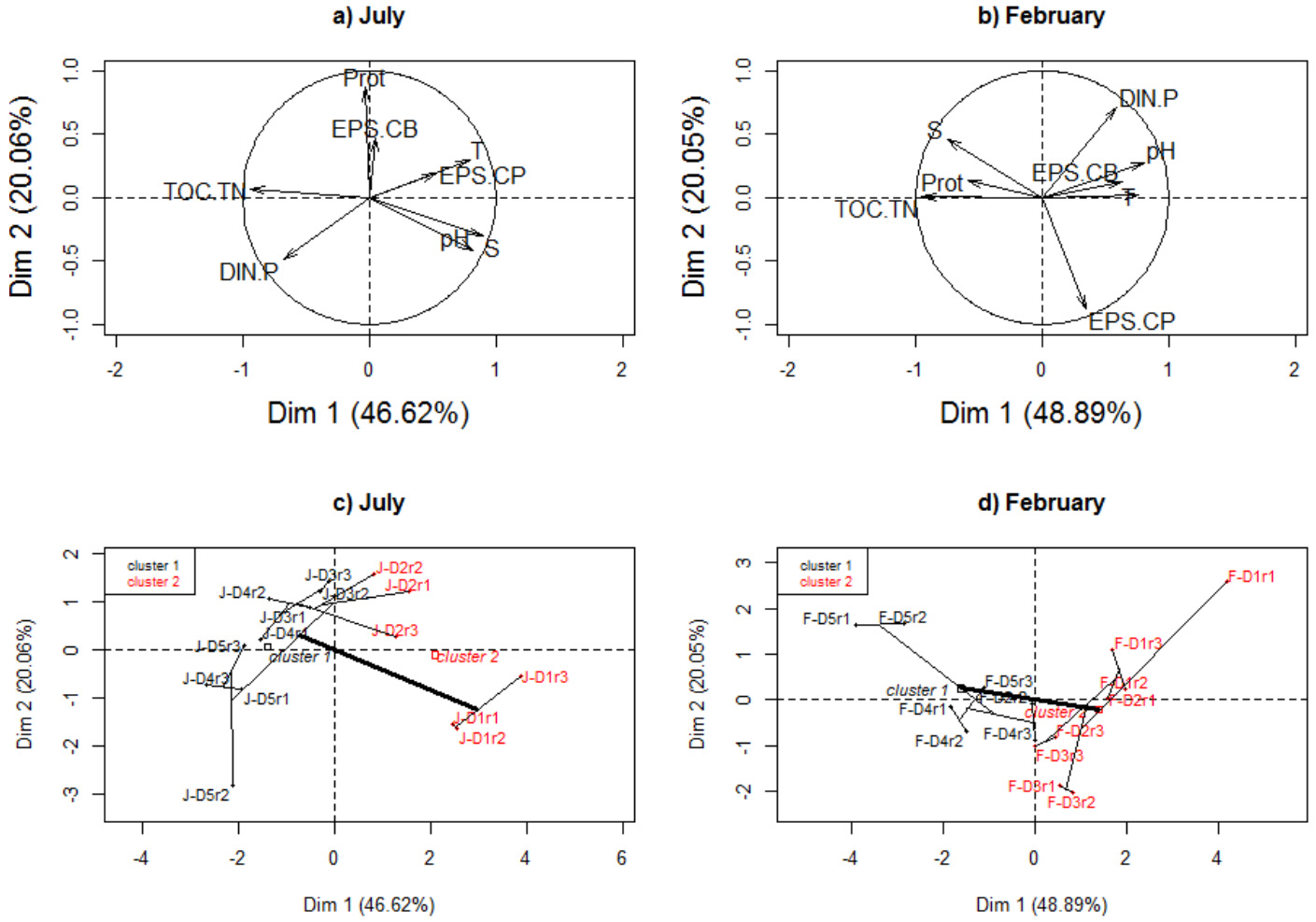


Figure 1. Principal component analysis (PCA) ordination calculated using 8 physico-chemical variables for a and c) 15 samples in July and b and d) 15 samples in February. a and b) Ordination of the variables and correlation circle. b and d) Position of the observations in the ordination; tree calculated hierarchical classification on principle components and the different clusters evaluated using 10000 iterations . T: temperature; S: salinity; Prot: total protein concentration; EPS.CB: colloidal EPS/bound EPS ratio; EPS.CP: EPS-carbohydrates/EPS-proteins ratio; DIN.P: DIN:PO_4^{3-} ratio; TOC:TN: ratio of total organic carbon (TOC) and total nitrogen (TN). PCA and hierarchical classification were performed using "FactoMineR" package (Husson et al., 2013).

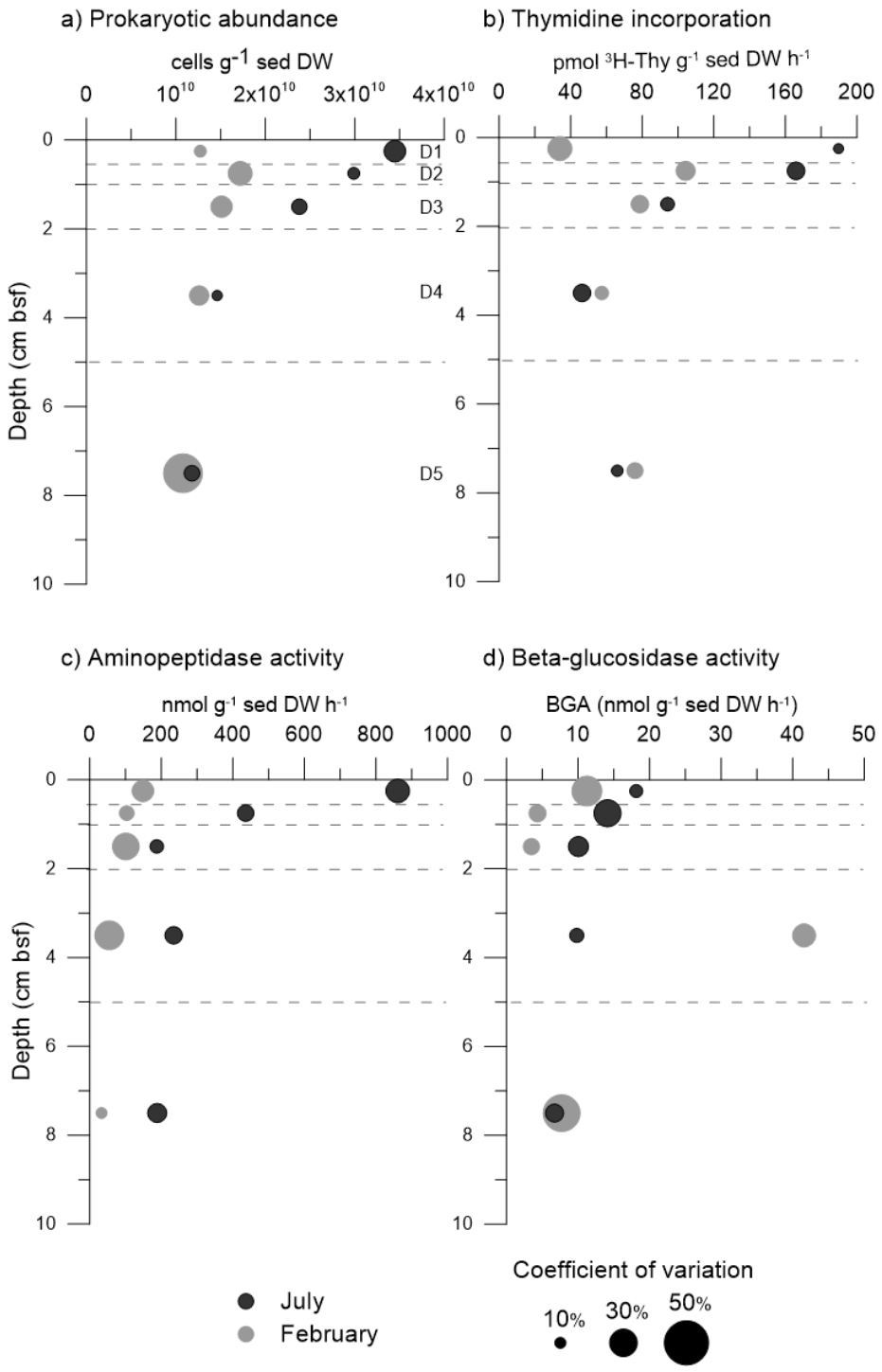
330 *5.2 Prokaryotic abundances and activities*

331 Prokaryotic abundances ranged from $1.18 \pm 0.20 \times 10^{10}$ to $3.45 \pm 1.05 \times 10^{10}$ cells g^{-1} sed DW
332 in July with maximum values in the surficial sediment layer (0-0.5 cm below the sediment
333 surface) (Figure 2). Abundances were significantly lower in February than in July (two-way
334 ANOVA, F=24.16, $p < 0.001$, Supp info Table S3) with values between $1.08 \pm 0.75 \times 10^{10}$ and
335 $1.72 \pm 0.50 \times 10^{10}$ cells g^{-1} sed DW and a peak recorded between 0.5 and 1 cm bsf. In July,
336 thymidine incorporation (a proxy for benthic bacterial production) decreased with depth from
337 189.69 ± 8.15 to 46.31 ± 9.93 pmol ^{3}H-Thy g^{-1} sed DW h^{-1}. In February, thymidine
338 incorporation was lower but showed a similar decrease with depth. For both sampling dates,
339 thymidine incorporation and prokaryotic abundance distribution profiles were very similar
340 (Pearson test, n=30r^2 = 0.806, $p < 0.001$) (Figure 2). The impact of a 10°C-increase on
341 thymidine production was expressed by using Q_{10}. The temperature had a strong impact on
342 thymidine production between 0 and 0.5 cm bsf (average value of Q_{10}=6.265). Then, between
343 0.5 and 1 cm bsf, temperature effect was less important (average value of Q_{10}=1.589) but
344 significantly different from 1 (t-test one sample, t= 3.4589, p = 0,009). Between 1 and 10 cm
345 bsf, the temperature had no effect as Q_{10} values were not significantly different from 1 (t-test
346 one sample, $p > 0.01$).

347 Variance analysis (two-way ANOVA) showed that potential aminopeptidase activity was
348 significantly higher in July (F=75.29, $p < 0.001$, Supp info Table S3) (mean for all depth: 381.31
349 ± 78.64 nmol g^{-1} sed DW h^{-1}) than in February (mean for all depth: 88.02 ± 13.60 nmol g^{-1} sed
350 DW h^{-1}) and that in July, these activities were significantly different in the surface sediment
351 compared to the deeper layers (Tukey HSD test, $p < 0.001$) (Figure 2). Potential β-glucosidase
352 activity was generally low throughout all the sediment depths. Values ranged from 6.71 ± 1.09

353 to 18.14 ± 1.42 nmol g^{-1} sed DW h^{-1} in July and from 3.49 ± 1.21 to 41.59 ± 8.32 nmol g^{-1} sed

354 DW h^{-1} in February (Figure 2).



355

356 **Figure 2.** Prokaryotic abundances, production and activities along a vertical depth
357 gradient below the sediment surface (bsf). All points represent the middle of each layer. The
358 coefficient of variation is displayed as bubble size. Black bubbles represent values for July 5,
359 2012, and gray bubbles represent values for February 11, 2013.

5.3 Algal biomass

The algal biomass on the surface (D1) was 69.5 ±2.4 µg Chl *a* g^{-1} sed DW 59.4 ±1.7 µg Chl *a* g^{-1} sed DW during the samplings in July and in February, respectively (Table 1). The highest standard errors were recorded in D1, resulting probably from the patchiness distribution of the microphytobenthos observed in the field. The algal biomass showed an exponential decrease with values never exceeding 17.40 µg Chl *a* g^{-1} sed DW under 0.5 cm bsf (Table 1).

5.4 The distribution of fauna abundances

The abundance of six meiofaunal groups was recorded: nematods, copepods, ostracods, small gastropods, small bivalves and foraminifera (Supp info Fig S5). The most abundant were the nematods (maximum abundance= 1060 ind cm^{-3}) and foraminifera (maximum abundance= 57 ind cm^{-3}). The abundances of groups investigated decreased with depth increase (Supp info Fig. S5). Higher abundances were recorded in July except for copepods and ostracods. Additionally, the macrozoobenthic grazer, *Peringia ulvae* (Pennant, 1777) present at the surface of the sediment appeared to be more abundant in February (1908 ind m^{-2}) than in July (528 ind m^{-2}).

5.5 Factors influencing prokaryotic activities and densities

All variables used in the variation partitioning analysis (E table: salinity, phosphate and silicate concentrations and M table: juvenile gastropods) had a significant effect on prokaryotic activity and abundance (Supp info Table S4). The environmental variables (E table) explained 14.25 % of the variance of distribution of the prokaryote-related variables without the component variations shared with the meiofauna abundance (M table). While meiofaunal abundances explained 5.72% of the variation of prokaryotic variables. Collectively, phosphate and silicate concentrations, salinity, and abundances of little gastropods explained 59% of the prokaryotic abundances and activity variations (Figure 3).

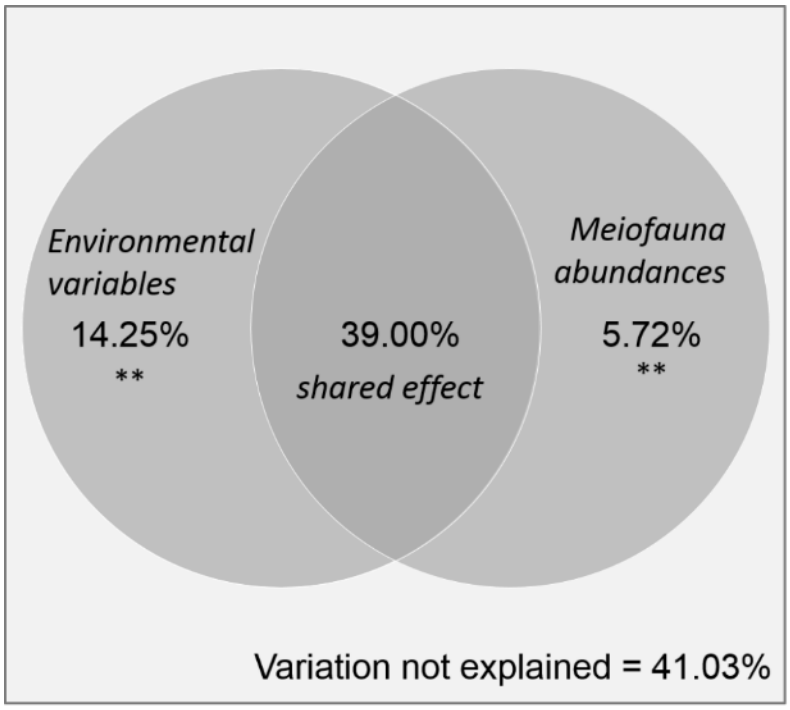


Figure 3. Venn diagram based on a variation partitioning from prokaryotic variables (*i.e.,* prokaryotic abundance; thymidine incorporation; aminopeptidase activity; and beta-glucosidase activity). The external square represents the whole variation of the prokaryotic table. Each circle represents the explanatory tables and values are the part of the variation explained by each explanatory table. The variables used in the analysis was previously selected by forward selection and final tables included: Environmental variable table: Salinity, PO_4^{3-} concentrations, and silicate concentrations; and meiofauna table: abundances of juvenile gastropods. Statistically significant pure fraction of variation of prokaryotes communities are presented as: <0.01 ** (ANOVA like permutation test, 9999 permutations) and details are given in Supp info Table S4.

6 Discussion

The muddy sediments in Marennes-Oléron Bay support high microbial activities and production rates as is typical for fine-grained sediments (Böer, 2008; Llobet-Brossa et al., 1998). This study shows depth gradients of prokaryotic abundances and activities in the top 10-cm of these coastal mudflats based on the analyses of depth layers chosen to characterize centimetre-scale processes. The stratification was particularly pronounced for the sampling in July and this appeared to be related to depth variation of abiotic and biotic environmental variables. Using the set of these environmental variables in the different depth layers, we have studied how they could statistically explain the differences of prokaryotic abundances and activities in the sediment. However, we had to exclude grain size and oxygen. The former showed hardly any variation with depth, while the latter is known to show mm-scale variation close to the surface that was not adequately measured in this study. Nevertheless, a sufficiently large panel of biotic and abiotic variables were available for disentangling the contributions of these environmental variables for driving prokaryotic abundances and activities.

6.1 Relative impact of environmental variables and meiofauna: the main driving factors

A forward selection identified that prokaryotic abundances and activities were significantly influenced by salinity, phosphate and silicate concentrations as well as juvenile gasteropod abundances. Above all, the resulting variation partition, underlined that the interaction among physicochemical variables and meiofaunal abundance is high and has a significant impact on prokaryotic abundances and activities (Figure 3). While the gasteropod juveniles are not abundant in this study, their distribution significantly affects the prokaryotic-related variables and is strongly related to physicochemical variables (*i.e.*, large part of variance explanation shared with environmental table).

417 Nitrites or nitrates are more often identified as forcing factors for prokaryotic
418 communities in sediments (Böer et al., 2009), however, in the current study, the use of variation
419 partition shows that others inorganic nutrients such as silicates and phosphates significantly
420 influenced the prokaryotic activities and abundances. Interestingly, the phosphate
421 concentrations appeared to limit the prokaryotic activities (*e.g.*, thymidine incorporation,
422 aminopeptidase and beta-glucosidase activities) more than nitrogen-related nutrients in bottom
423 layers in July and in surface in February (*i.e.* DIN:PO_4^{3-} ratio>16; Supp info Figure S2).
424 In a previous study, Pascal et al. (2009) showed that only 6 % of the total bacterial
425 biomass was controlled by consumers in the first 1 cm of the sediment surface, suggesting a
426 major effect of resources in the Marennes-Oléron mudflat. Our statistical results suggest that
427 the activities and abundances of benthic prokaryotes in the first 10 cm of sediment were more
428 influenced by physicochemical properties of the sediment *(i.e.*, inorganic nutrients and salinity)
429 rather than by predation pressure by meiofauna (Supp info Table S4). The variation partitioning
430 that we propose statistically identifies that bottom-up control (represented by physicochemical
431 variables) had stronger influence on prokaryotic activities than top-down control by meiofauna
432 and that the shared interactions between the two trophic controls are of major importance. In
433 the current study, it appears that physicochemical properties of the sediment that varied with
434 depth strongly stratified the biotic communities. The high part of variation explained by the two
435 trophic controls could reflects this influence of physicochemical variables on both prokaryotic
436 activities and abundances and meiofauna abundances. However, it could also be due to the fact
437 that meiofauna could slightly modifies the vertical stratification of organic matter, inorganic
438 nutrient or EPS composition. Other proxies can be used to identify factors that drive microbial
439 communities. For example, Pace & Cole (1994) proposed that a strong positive correlation
440 between prokaryotic biomass and production rates indicates bottom-up control. This relation

441 can thus be successfully applied to understand the relationships in benthic microbial ecology,
442 although other factors such as organic matter should also be considered.

443 *6.2 Two horizons, two different stories*

444 The principal component analysis followed by hierarchical clustering based on
445 physicochemical variables confirmed a vertical zonation mainly described by organic matter
446 composition (*i.e.,* the TOC:TN ratio), pH and salinity (Figure 1). Collectively, our results
447 showed that the upper 10 cm of the sediment was divided into two clearly different horizons
448 with thickness varying between the samplings in July and February. The surface horizon,
449 separated from the bottom one by a transition layer is thicker in February (2 cm) than in July (1
450 cm). The position of the transition zone proposed here was therefore dependent on the thickness
451 of the sampling layers in our study and was expected to fluctuate from 1 cm to 3.5 cm bsf
452 (middle of our sampling layer).

453 The biotic and abiotic variables in the surface horizon differed between the two
454 sampling dates. In July, prokaryotic and environmental variables (*e.g.,* prokaryotic abundance,
455 thymidine incorporation, aminopeptidase activity, EPS-carbohydrates and salinity) were high
456 compared to February (Table 1 and Figure 2). For example, in July, aminopeptidase activity
457 was particularly high compared to other studies (as reviewed by Danovaro et al. (2002)) but
458 comparable with aminopeptidase activities recorded in the Balearic Sea (Tholosan et al., 1999).
459 Thymidine incorporation, used as a proxy of benthic bacterial production, drastically increased
460 with an increase of 10°C (*i.e.*, high Q_{10} value). On the basis of our results (Figure 2), we
461 hypothesized that in February, in the surface horizon (0-2 cm bsf), the prokaryotic communities
462 showing low metabolic activities were not able to sustain growth as a large part of their
463 metabolic energy was used for maintenance. In contrast, in July, as a result of higher
464 temperature, the high densities and high metabolic rates of prokaryotes seemed to be related to
465 metabolically active and growing populations. At low tide, prokaryotic populations in the

surface horizon are strongly influenced by external parameters (*e.g.*, light exposure, resuspension and tidal cycle) and microphytobenthic activity. Although algal biomass (*i.e.*, as measured by chlorophyll *a* concentration) was in the same range for both sampling dates, the high microphytobenthic primary production in July (gross primary production: 6.0 ± 1.7 mg C h^{-1} m^{-2}, CO_2 fluxes in benthic chambers measurement method, pers. comm. from J. Lavaud) had probably enhanced the bacterial production in the sediment top layer (0-0.5 cm bsf). This source of labile carbon may be quickly transferred to the bacterial compartment as shown previously in sandy sediments (Cook et al., 2007) and intertidal flats (Middelburg et al., 2000). Moreover, large amounts of EPS-carbohydrates were recorded in July compared to February and these EPS may be produced by epipelic diatoms in response to nutrient limitation or photo-protection (Smith and Underwood, 2000, 1998). Together, the high EPS-carbohydrates concentrations, the low nutrient concentrations, and the DIN:PO_4^{3-} ratio below the Redfield value (Redfield, 1958), suggested a nitrogen limitation for benthic micro-organisms in surface in July.

While EPS-carbohydrates were dominant in July, EPS-proteins clearly increased in February (as shown by the shift of the ratio EPS-carbohydrates/EPS-proteins, Supp info Figure S3). At this date, both prokaryotic density and thymidine incorporation were low in the top horizon (0-2 cm bsf, Figure 2) and this was not only due to the low sediment temperature because higher bacterial production occurred deeper in the sediment despite a similar temperature. A study in Marennes-Oléron mudflat (Orvain et al., 2014b) shows that a higher proportion of EPS-proteins coincided with mass erosion events and higher abundance of the macrozoobenthic grazer, *Peringia ulvae* (Pennant, 1777). These macrozoobenthic grazers may disturb the sediment stability by grazing on biofilm and EPS-proteins may potentially originated from shell mucus (Orvain et al., 2014b). Based on these features and on our results, it may be possible that the highest abundance of *Peringia ulvae* (Pennant, 1777) recorded in

491 February provoked a high predation pressure (*i.e.*, predation pressure: 1.72 mg C h^{-1}, calculated
492 according to Pascal et al. (2009)) and an increase of EPS-proteins, hence inducing mass erosion
493 of the sediment. This erosion is associated with the release of diatoms and prokaryotes into the
494 water column (Guizien et al., 2014; Montanié et al., 2014; Shimeta et al., 2002) and may
495 therefore impact the surface of sediment in February by a decrease of prokaryotic density and
496 bacterial production. Finally, in our study, even if mass erosion of the sediment surface might
497 have occurred at seeing the sea state (Suppl. Info, Table S2) and the wind speeds (data not
498 shown), prokaryotic abundance could be lower because of the grazing of *Peringia ulvae*
499 (Pennant, 1777) or by viral lysis that has been reported to be responsible for the loss of 40 % of
500 bacterial production in Marennes-Oléron mudflat (Saint-Béat et al., 2013). These results
501 suggesting a mass erosion event that occurred in February are consistent with a thicker surface
502 horizon (from 0 to 2 cm bsf) compared to the one in July.

503 In the bottom horizon, between 1 or 2cm bsf (in July and February, respectively) and 10
504 cm bsf, all biotic and physicochemical gradients showed little variation with depth. For both
505 sampling dates, the thymidine incorporation used as a proxy of bacterial production was similar
506 below 2 cm depth despite high environmental differences. Indeed, temperature, salinity, and
507 the EPS-carbohydrates strongly decreased from July to February, and nutrient concentrations
508 also changed—specifically, phosphate and ammonium concentrations increased (Supp info
509 Figure S2). While this bacterial production was clearly lower in this bottom horizon compared
510 to the surface one we probably underestimated thymidine uptake in the anoxic layers because
511 the experiments were not performed under anoxic conditions while microorganisms may be
512 partially or strictly anaerobes. Despite this potential underestimation, bacterial communities
513 were able to maintain the same production level between 2 and 10 cm bsf in both sampling
514 dates, suggesting that the system may potentially contain a low and stable microbial bulk
515 activity in this horizon throughout the year independently of environmental changes.

6.3 The transition zone

The boundary layer may represent a transition zone between the surface horizon largely influenced by external parameters and the bottom horizon corresponding to reduced sediment. The current study proposes that the transition zone should represent the limit of influence of weather conditions on sediment physicochemical properties and thus on prokaryotic activities in the intertidal mudflat. The depth of this layer was expected to fluctuate weakly over the seasons and among the low tide period. Notably, storms can destroy the vertical structure deeper than the external parameter-influenced zone. Nevertheless, except during these rare but strong events, the depth of this surface layer can be considered specific to intertidal muddy sediments. Indeed, sandy sediments are generally permeable and allow advective fluxes of water through the interstitial spaces (Musat et al., 2006) and thus exhibit a different depth profile compared to muddy sediments. Except for transient storms, the transition layer is thus located at 1-3.5 cm depth in intertidal muddy sediments.

Whether this transition zone represents an environmental ecotone or ecocline can be discussed. These two terms have been largely used in ecology to characterize boundary zones where gradients occur, but their definitions and how to use them are still unclear (Erdôs et al., 2011). Nevertheless, many authors agree that the term environmental ecotone defines a gradient between two adjacent habitats characterized by rather abrupt changes and that it comprises habitats that should be very specific for certain species (Attrill and Rundle, 2002; Erdôs et al., 2011; van der Maarel, 1990; Whittaker, 1967). In contrast, an environmental ecocline stands for more gradual changes that may result from mixing of the two communities from the neighboring habitats (Attrill and Rundle, 2002; Erdôs et al., 2011; van der Maarel, 1990; Whittaker, 1967). In the present study, the transition zone corresponded to a gradient zone at a cm scale which we characterized by a gradual change of environmental variables such as porosity or EPS ratios and a gradual change of microbial communities (*e.g.*, algal biomass,

enzymatic activities and prokaryotic abundance). Hence, following these definitions and our findings, we should rather consider the identified transition zone as an environmental ecoclinal boundary (Erdôs et al., 2011).

6.4 Conclusion

The current study provided detailed snapshots of the depth gradients of prokaryotic abundances and process rates at two sampling dates at low tide. The detailed stratification pattern using a large ensemble of variables and different multivariate analyses allowed us to decipher some of the major factors driving the densities and activities of microbial populations in intertidal sediments. Thus, we succeeded in statistically explaining a large part of the prokaryotic activity distributions by the environmental variables (*i.e.*, salinity and nutrients), and to a lesser extent by consumers (meiofauna), suggesting that bottom-up control was more important than top-down control. In general we observed that the top 10 cm of these muddy sediments comprise two clearly different depth horizons that are separated by a transition zone. Thus we identified a surface horizon, which appears variable in thickness between sampling dates and where prokaryotic activities and densities are highly impacted by microphytobenthic activities and physicochemical variables and, a deeper and more stable bottom horizon. The transition appears to be gradual corresponding to an environmental ecocline rather than an ecotone.

Nevertheless, one part of this distribution remained statistically unexplained (41% of the variation is estimated to be unresolved by the chosen variables in the variation partitioning) and further studies are needed to explore 1) other abiotic variables such as sulfate, iron oxide or manganese oxide concentration, 2) prokaryotic activity and production dynamics throughout the low tide period, and 3) other prokaryotic indices such as diversity or functional genes.

7 Figure captions

Figure 1. Principal component analysis (PCA) ordination calculated using 8 physico-chemical variables for a and c) 15 samples in July and b and d) 15 samples in February. a and b) Ordination of the variables and correlation circle. b and d) Position of the observations in the ordination; tree calculated hierarchical classification on principle components and the different clusters evaluated using 10000 iterations . T: temperature; S: salinity; Prot: total protein content; EPS.CB: colloidal EPS/bound EPS ratio; EPS.CP: EPS-carbohydrates/EPS-protein ratio; DIN.P: DIN:PO_4^{3-} ratio; TOC:TN: ratio of total organic carbon (TOC) and total nitrogen (TN). PCA and hierarchical classification were performed using "FactoMineR" package (Husson et al., 2013).

Figure 2. Prokaryotic abundances, production and activities along a vertical depth gradient below the sediment surface (bsf). All points represent the middle of each layer. The coefficient of variation is displayed as bubble size. Black bubbles represent values for July 5, 2012, and gray bubbles represent values for February 11, 2013.

Figure 3. Venn diagram based on a variation partitioning from prokaryotic variables (*i.e.*, prokaryotic abundance; thymidine incorporation; aminopeptidase activity; and beta-glucosidase activity). The external square represents the whole variation of the prokaryotic table. Each circle represents the explanatory tables and values are the part of the variation explained by each explanatory table. The variables used in the analysis was previously selected by forward selection and final tables included: Environmental variable table: Salinity, PO_4^{3-} content, and silicate content; and meiofauna table: abundances of juvenile gastropods. Statistically significant pure fraction of variation of prokaryotes communities are presented as: <0.01 ** (ANOVA like permutation test, 9999 permutations) and details are given in Supp info Table S4.

8 Table caption

Table 1. Average of each environmental variables and algal biomass (± SE) along sediment depths. Two-way ANOVA reveals always significant differences between the two sampling dates for all variables ($p < 0.05$), letters in bold font indicate Tukey's post hoc test for each sampling date.

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