Heterotrophic activities of neustonic and planktonic bacterial communities in an estuarine environment (Ria de Aveiro)

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The uppermost millimeter of the water column, the surface microlayer (SML), hosts bacterial communities (bacterioneuston) with potential metabolic adaptations to this unique physical and chemical environment. Hydrolysis and monomer incorporation by bacterioneuston and bacterioplankton communities in the estuarine system, Ria de Aveiro, was investigated and compared during a 2-year survey. The study was conducted at two contrasting sites, typifying the marine and brackish water characteristics of the estuary and with different sources, amounts and composition of organic matter. In the marine zone, bacterioneuston showed higher rates of hydrolysis and lower rates of monomer incorporation than bacterioplankton, whereas in the brackish water zone, neustonic and planktonic microbial communities showed similar activity rates. This pattern may result from the different degree of surface organic and inorganic matter enrichments, which reflect site-specific characteristics, such as hydrodynamics and sources of organic matter composition. In general, the estuarine SML environment favors polymer hydrolysis, but inhibits monomer utilization, in comparison with the subsurface layers. However, the differences between the two communities tend to decrease as autotrophic and heterotrophic activities increase in the brackish-water area.

KEYWORDS: surface microlayer; bacterioneuston; bacterioplankton; bacterial monomer incorporation; bacterial ectoenzymatic activity; estuary; Ria de Aveiro
INTRODUCTION

Located at the air–water interface of potentially all aquatic systems, surface microlayers (SMLs) are unique microbial habitats, where neustonic microorganisms interact with the atmosphere and the hydrosphere (Cunliffe et al., 2011). The SML comprises the top 1000 μm of the water column (Liss and Duce, 1997) and is physical and chemically distinct from the underlying water (UW) (Zhang et al., 2003). Diverse physical processes, such as diffusion, turbulent mixing, scavenging and transport by bubbles and buoyant particles (Liss et al., 1997), contribute to the concentration of numerous organic and inorganic compounds at the SML (Sieburth et al., 1976; Henrichs and Williams, 1985; Williams et al., 1986; Hunter, 1997a; Liss and Duce, 1997), most of which originates in the water column (Liss and Duce, 1997; Kuznetsova et al., 2004). Increasing evidence supports the concept that the SML is a gelatinous biofilm (Sieburth, 1983; Wurl and Holmes, 2008; Cunliffe and Murrell, 2009), where colloidal material (Bigg et al., 2004) and transparent exopolymer particles (TEPs) are enriched (Kuznetsova et al., 2003; Cunliffe et al., 2009; Wurl et al., 2009).

The organic enrichment of the SML likely favors heterotrophic activity (Obernosterer et al., 2005), fueling highly active communities of microorganisms (Sieburth et al., 1976). Microbial dissolved organic matter (DOM) transformation processes, such as enzymatic hydrolysis (Munster et al., 1998; Kuznetsova and Lee, 2001; Mudryk and Skórzewska, 2004) and uptake (Carlucci et al., 1991; Garabéïtan, 1991; Munster et al., 1998; Santos et al., 2009), are enhanced at the SML compared with in the water column probably due to the greater concentrations of DOM at this interface (Kuznetsova and Lee, 2001). Despite a potentially higher availability of substrates at the SML, bacterioneuston growth efficiencies were found to be very low, suggesting low accessibility of substrates for bacteria at this interface (Reinthaler et al., 2008). Therefore, the activity of bacteria at the SML might reflect chemical and physical processes that contribute to the organic enrichment at the air–water interface and to organic matter accessibility for bacterial utilization.

Organic matter in estuaries is derived from multiple inherent characteristics, such as wind (Stolle et al., 2010) and water circulation (Santos et al., 2011), change the dynamic of the SML, influencing the distribution and activity of microorganisms in the SML and in the water column and, therefore, the study of organic matter transformation in these ecosystems is challenging.

This work aimed to determine and compare seasonal and spatial variations of heterotrophic activities of neustonic and planktonic microbial communities in an estuarine system. To achieve this goal, two contrasting zones of the Ria de Aveiro estuary, with different amounts and sources of organic matter and differently impacted by estuarine hydrodynamic characteristics, were chosen. This study is extensive, in terms of study period and variety of monomers and enzymatic activities investigated, and provides insight into microbial organic matter transformation in the SML.

METHOD

Study site

Ria de Aveiro (40° 38’ N, 8° 45’ W; Fig. 1) is a shallow tidal lagoon situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand barrier. The lagoon covers an area of 66 and 83 km² at low and high tide, respectively. It exchanges a volume of water of 137 Mn³ with the sea at maximum spring tide and 35 Mn³ for minimum neap tide (Dias et al., 2000). Several rivers carry fresh water into the lagoon with an average water input of 1.8 Mn³ during a tidal cycle (Dias et al., 2003). The lagoon has a complex topography, with four main channels (Espinheiro, S. Jacinto, Mira and Ilhavo) spreading from the mouth in diverse streams, forming a complex estuarine system (Dias et al., 2001). For this study two sampling stations were selected: station N1, located at the entrance of the estuary, representing the marine and deep (average depth at low tide, 26 m) conditions, and station 16, located in the Ilhavo channel, representing the brackish water and shallow (average depth at low tide − 2 m) conditions of estuary.

Sampling

Sampling was carried out at low tide, approximately every 2 months during 2006 and 2007. SML samples were collected with a Plexiglas plate (Harvey and Burzell, 1972), which collects roughly the upper 60–100 μm water layer. The plate dimensions were 0.25 m wide × 0.35 m long and 4 mm thick. Before sampling, the plate was cleaned with ethanol, rinsed with sterile distilled
water and finally with water from the sampling site. The water adhering to the plate during immersion was removed from both sides forcing the plate between two Teflon sheets and collecting the water into a sterilized glass bottle. Approximately 1 L of SML water was collected at each sampling point. Samples from UW were collected with a horizontal Van Dorn bottle at 20 cm depth and transferred to a sterilized glass bottle. Samples were kept cold during transport to the laboratory and processed within 2–3 h of collection.

Environmental parameters

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstätten, Germany), and dissolved oxygen concentration was measured with a WTW OXI 320 oxygen meter (Wissenschaftlich Technische Werkstätten, Germany). Temperature and dissolved oxygen concentration were only determined for UW because the collection of the SML takes a considerable time during which changes in temperature and oxygen concentration are likely to occur.

Chlorophyll a (Chl a) concentration was estimated fluorimetrically (Yentsch and Menzel, 1963) after filtration of 0.5 L triplicate subsamples through Whatman GF/F filters and overnight cold extraction in 90% (v/v) acetone. Suspended particulate matter (SPM) concentration was determined after filtration of triplicate 0.5-L water aliquots through pre-weighed and pre-combusted Whatman GF/F filters. The filters were dried at 60°C for 24 h, and SPM was calculated as the increase in dry weight. Particulate organic matter (POM) was determined from the further decrease in weight after 4 h incineration at 550°C (Parsons et al., 1989). For nutrient analysis, water subsamples were filtered through MSI acetate membranes with 0.45-μm pore size and stored at −20°C in acid-cleaned polyethylene flasks until determination. Orthophosphate and nitrite were quantified using methods described by Hansen and Koroleff (Hansen and Koroleff, 2007). Nitrate was assayed using an adaptation of the spongy cadmium reduction technique (Jones, 1984), with the nitrite value subtracted from the total. Temperature and dissolved oxygen concentration were only determined for UW because the collection of the SML is a slow process during which changes in temperature and oxygen concentration are likely to occur. Furthermore, the SML collection with the Plexiglass plate sampler is a time-consuming process, also retrieves very small amounts of water, and for that reason, the concentrations of Chl a, SPM, POM, dissolved organic carbon (DOC) and nutrients were also only determined for UW.

Meteorological parameters

Wind speed data coincident with the sampling period were recorded at the University of Aveiro meteorological station, located close to the sampling area (http://climetua.fis.ua.pt/).

Microbiological parameters

The number of total and particle-attached bacteria (PAB) was determined by epifluorescence microscopy using a
Leica “DMLS” microscope equipped with a I 2/3 filter for blue light. Particle-attached cells were counted directly and distinguished from free-living cells on the same slide. Three replicates for each sample were filtered through 0.2-μm black polycarbonate membranes (GE Osmonics) and stained with 0.03% acridine orange (Hobbie et al., 1977). At least 200 cells or 20 microscope fields were counted for each replicate measurement.

The heterotrophic metabolism of the 14C-labeled monomer acetate (SA 2.04 GBq mmol⁻¹, 55.0 m Ci mmol⁻¹), glucose (SA 11.5 GBq mmol⁻¹, 310 m Ci mmol⁻¹) and leucine (SA 11.3 GBq mmol⁻¹, 306 mCi mmol⁻¹) was described by the parameter Vm (maximum uptake velocity) following the procedure described by Gocke (Gocke, 1977). A final saturation concentration of 430 nM of each radioactively labeled substrate was added to 10 mL aliquots of the water samples. Substrate concentrations were chosen after kinetic analysis. Incubations were carried out for 2 h at in situ temperature. Cells were collected on 0.2-μm pore-size filters (GE Osmonics) and radioactivity was read in a liquid scintillation counter (LS 6000 IC, Beckman) using UniverSol (ICN Biomedicals) as scintillation cocktail. Radioactively labeled acetate, glucose and leucine were obtained from Amersham.

Extracellular enzymatic activity was determined fluorimetrically (Jasco FP-777 fluorometer) as the maximum hydrolysis rate (Hm) of model substrates (Hoppe, 1991). The following substrates were used for the indicated enzyme (substrate for enzyme): t-leucine-7-amido-4-methylcoumarin hydrochloride (Fluka) for leucine aminopeptidase (E.C. 3.4.11.1) (Kanaoka et al., 1977); 4-methylumbelliferyl-β-glucopyranoside (Fluka) for β-glucosidase (E.C. 3.2.1.21) (Daniels et al., 1981); 4-methylumbelliferyl-β-D-galactopyranoside (Sigma) for β-D-galactosidase (EC 3.2.1.23) (Vernet et al., 1993), 4-methylumbelliferyl-fosfate (Sigma) for alkaline phosphatase (APase) (E.C. 3.1.3.1) (Roberts et al., 1991); 4-methylumbelliferyl-acetate (Sigma) for esterase (E.C. 3.1.1.2); 4-methylumbelliferyl-palmitate (Sigma) for lipase (E.C. 3.1.1.3) (Gajewski et al., 1997). The substrates were added at saturating concentrations (10 mM of acetate, 4-methylumbelliferyl-β-glucopyranoside, t-leucine-7-amido-4-methylcoumarin hydrochloride and 4-methylumbelliferyl-fosfate; 4 mM of 4-methylumbelliferyl-palmitate and 4-methylumbelliferyl-β-D-galactopyranoside). Wavelengths for excitation and emission were 380–440 nm for MCA (7-amino-4-methylcoumarine) and 360–450 nm for MUF (4-methylumbelliferone). Measurements were made in three replicates for each sample after 2 h, for MCA, and 18 h for MUF. Incubations were made at in situ temperature. Calibration was performed by adding a series of 6–8 concentrations of the fluorescent products (0–500 nM for MUF and 0–6 μM for MCA) to a pool of water from the two sampling stations. Extracellular enzymes play a central role in heterotrophic microbial cycling of carbon, and the determination of their activity is a robust tool for obtaining comparative insight into organic matter decomposition and microbial activity in aquatic ecosystems (Chroš, 1992). These enzymes enable the utilization of substances that otherwise would not be directly accessible to microorganisms (Chroš, 1992), by catalyzing the initial step of high-molecular-weight organic matter conversion to substrates small enough to be transported into the intracellular compartment (~600 Da) for further processing (Arnosti, 2011).

Data analysis

Microbiological parameters determined for SML and UW were compared and expressed as the enrichment factor (EF) defined as EF = [X]SML/[X]UW where [X] is the concentration of a given parameter (Gesamp, 1995). The statistical analysis of microbiological data was performed with the SPSS 15.0 (SPSS Statistics) software. One-way ANOVA was used to determine the significance of the differences observed in microbial parameters between the two layers. Normal distribution was assessed by the Kolmogorov–Smirnov test and the homogeneity of variances by the Levene test. A value of P < 0.05 was considered significant.

RESULTS

Physical and chemical parameters

The values of the physical and chemical parameters determined in the SML and in UW are shown in Table I. Salinity was similar, averaging 28.3 ± 8.56 at station N1 and 20.0 ± 12.01 at station I6. Temperature showed a typical seasonal variation, averaging 16.0 ± 2.56°C at station N1 and 17.5 ± 5.21°C at station I6. Oxygen concentration was similar in both zones, averaging 7.2 ± 3.3 mg L⁻¹.

Meteorological parameters

The average daily wind speed (Table I) during the sampling, varied between 1.0 and 40.3 m s⁻¹ (average 13.1 ± 12.4 m s⁻¹), and was eight times more intense during 2006 compared with 2007.

Characteristics of the UW

The values of the concentration of Chl a, suspended (SPM) and particulate matter (POM), DOC and nutrients determined in bulk water are shown in Table II. Chlorophyll a
concentration followed a typical seasonal pattern, averaging $2.9 \pm 1.6 \mu g \, L^{-1}$ in the marine zone and $4.9 \pm 2.7 \mu g \, L^{-1}$ in the brackish water zone. SPM and POM concentrations were similar in both zones, averaging $55.0 \pm 16.20 \, mg \, L^{-1}$ and $13.1 \pm 5.24 \, mg \, L^{-1}$, respectively. DOC averaged $3.1 \pm 6.79 \, mg \, L^{-1}$ in the marine zone and $10.0 \pm 6.79 \, mg \, L^{-1}$ in the brackish water zone.

The concentration of nitrates plus nitrites ($NO_3^- + NO_2^-$) averaged $7.4 \pm 5.0 \, mM$ in the marine zone and $29.1 \pm 23.7 \, mM$ in the brackish water zone. Phosphate concentration was, on average, $2.6 \pm 4.5 \, mM$ in the marine zone and $5.7 \pm 11.8 \, mM$ in the brackish water zone.

**Total and particle-attached bacterial numbers**

Total bacterial numbers (TBNs) in the SML and UW were similar (ANOVA; $P > 0.05$) both in marine (average $EF = 1.2$) and brackish water (average $EF = 1.3$) zones, varying between 0.5 and $20.5 \times 10^9 \, cells \, L^{-1}$ (Table III). Nevertheless, the numbers of PAB were significantly enriched in the SML (average $EF = 4.6$; ANOVA, $P < 0.05$) of both estuarine zones, ranging from 0.03 to $19.7 \times 10^9 \, cells \, L^{-1}$. The fraction of bacteria attached to particles was enriched in the SML (two to three times), representing on average, $15.5\%$ at station N1 and $29.5\%$ at station I6.

**Bacterial heterotrophic activities**

The variation profiles of the maximum rates of monomer incorporation ($V_m$) are shown in Fig. 2. The highest $V_m$ values of leucine ($30.9 \, nmol \, L^{-1} \, h^{-1}$), glucose ($84.0 \, nmol \, L^{-1} \, h^{-1}$) and acetate ($465.6 \, nmol \, L^{-1} \, h^{-1}$) incorporation were observed in the brackish water zone of the estuary. In the marine zone, $V_m$ of leucine (average $EF = 0.2$), glucose (average $EF = 0.3$) and acetate (average $EF = 0.3$) incorporation by bacterio-neuston was always significantly lower (ANOVA, $P < 0.05$) than in bacterioplankton (Table IV). In the brackish water zone, leucine (average $EF = 1.0$), glucose (average $EF = 0.8$) and acetate (average $EF = 1.1$) incorporation $V_m$ values in the SML were similar to those observed in the UW (ANOVA, $P > 0.05$) (Table IV).

**Rates of polymers hydrolysis**

**Aminopeptidase activity**

The enzyme aminopeptidase (Leu-AMPase) showed the highest rates of activity at both sites of the estuarine system (Table IV; Fig. 3). $H_m$ of this enzyme varied between $420.1$ and $6489.2 \, nmol \, L^{-1} \, h^{-1}$ and the highest values were observed in the brackish water zone. The activity of Leu-AMPase was similar (ANOVA, $P > 0.05$) and

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**Table I: Physical and chemical determined in the surface microlayer (SML) and underlying water (UW) of the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro, and wind speed during the sampling events**

<table>
<thead>
<tr>
<th>Year</th>
<th>2006</th>
<th>2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month</td>
<td>Jan</td>
<td>Mar</td>
</tr>
<tr>
<td>Marine zone (N1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SML</td>
<td>34.0</td>
<td>23.6</td>
</tr>
<tr>
<td>UW</td>
<td>32.9</td>
<td>23.3</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>12.7</td>
<td>15.0</td>
</tr>
<tr>
<td>Oxygen (mg L$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>10.5</td>
<td>9.1</td>
</tr>
<tr>
<td>Brackish water zone (I6)</td>
<td></td>
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</tr>
<tr>
<td>Salinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SML</td>
<td>20.8</td>
<td>7.9</td>
</tr>
<tr>
<td>UW</td>
<td>20.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td></td>
</tr>
<tr>
<td>UW</td>
<td>11.0</td>
<td>16.7</td>
</tr>
<tr>
<td>Oxygen (mg L$^{-1}$)</td>
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<td></td>
</tr>
<tr>
<td>UW</td>
<td>12.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Wind intensity (m s$^{-1}$)</td>
<td>11.4</td>
<td>40.3</td>
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</table>

n.d., not determined.
Table II: Concentration of chlorophyll a, suspended particulate matter (SPM), particulate organic matter (POM), dissolved organic carbon (DOC), nitrate + nitrite (NO$_3$ + NO$_2$) and phosphate (PO$_4^{3-}$) in the underlying water (UW) of the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro

<table>
<thead>
<tr>
<th>Year</th>
<th>2006</th>
<th>2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month</td>
<td>Jan</td>
<td>Mar</td>
</tr>
<tr>
<td>Marine zone (N1) Chlorophyll a (µg L$^{-1}$)</td>
<td>2.4 ± 0.12</td>
<td>2.0 ± 0.12</td>
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<tr>
<td>SPM (mg L$^{-1}$)</td>
<td>46.6 ± 0.96</td>
<td>41.5 ± 1.89</td>
</tr>
<tr>
<td>POM (mg L$^{-1}$)</td>
<td>11.4 ± 0.50</td>
<td>10.5 ± 0.43</td>
</tr>
<tr>
<td>NO$_3$ + NO$_2$ (µM)</td>
<td>2.9</td>
<td>9.8</td>
</tr>
<tr>
<td>PO$_4^{3-}$ (µM)</td>
<td>11.3</td>
<td>7.6</td>
</tr>
<tr>
<td>SPM (mg L$^{-1}$)</td>
<td>3.0 ± 0.13</td>
<td>4.8 ± 0.12</td>
</tr>
<tr>
<td>POM (mg L$^{-1}$)</td>
<td>14.6</td>
<td>12.6</td>
</tr>
<tr>
<td>NO$_3$ + NO$_2$ (µM)</td>
<td>39.9</td>
<td>60.0</td>
</tr>
<tr>
<td>PO$_4^{3-}$ (µM)</td>
<td>1.3 ± 0.25</td>
<td>1.4 ± 0.12</td>
</tr>
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<td>n.d., not determined.</td>
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</table>

Table III: Total bacterial number (TBNs), particles-attached bacteria (PAB) and the fraction of particles-attached bacteria (%PAB) at the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro

<table>
<thead>
<tr>
<th></th>
<th>TBN (x 10$^9$ cells L$^{-1}$)</th>
<th>PAB (x 10$^9$ cells L$^{-1}$)</th>
<th>%PAB</th>
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<tr>
<td></td>
<td>SML</td>
<td>UW</td>
<td>EF</td>
</tr>
<tr>
<td>Marine zone (N1)</td>
<td>2.5 ± 1.97 (n = 11)</td>
<td>2.5 ± 2.55 (n = 11)</td>
<td>1.2 ± 0.39 (n.s.)</td>
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<tr>
<td>Brackish water zone (I6)</td>
<td>5.9 ± 5.17 (n = 11)</td>
<td>4.5 ± 3.10 (n = 11)</td>
<td>1.3 ± 0.42 (n.s.)</td>
</tr>
</tbody>
</table>

Average ± standard deviation.
EF, enrichment factor.
*ANOVA, P < 0.01.
**ANOVA, P < 0.05.
n.s., not significant (ANOVA, P > 0.05).
correlated in the SML and UW in both marine (average EF = 1.0) and brackish water (average EF = 1.3) zones.

**β-Glucosidase and β-galactosidase activities**

β-Glucosidase (β-GlCase) and β-galactosidase (β-GalCase) Hm ranged from 3.6 to 2071.0 nmol L$^{-1}$ h$^{-1}$ and from 1.1 to 369.0 nmol L$^{-1}$ h$^{-1}$, respectively (Table IV; Fig. 3). The highest Hm values of both enzymes were observed in the brackish water zone. β-GlCase potential activity was, on average, seven times higher (ANOVA, P < 0.05) in the SML compared with UW in the marine zone and similar (average EF = 1.3; ANOVA, P > 0.05) in the brackish water zone. β-GalCase Hm was, on average, 3.6 and 2.6 times higher (ANOVA, P < 0.05) in the SML than in the UW in the marine and brackish water zones, respectively.

**Phosphatase activity**

APase Hm ranged from 7.2 to 727.4 nmol L$^{-1}$ h$^{-1}$ and the highest value was observed in the SML of the marine zone (Table IV; Fig. 4). Here, the activity of this enzyme was, on average, 6.6 times higher (ANOVA, P < 0.05) in the SML than in the UW. In the brackish water zone, APase Hm was also significantly higher in the SML environment (average EF = 2.1; ANOVA P < 0.05).

**Esterase**

Esterase Hm varied between 2.6 and 1840.1 nmol L$^{-1}$ h$^{-1}$ and the highest value was observed in the brackish water zone (Table IV; Fig. 4). On average, potential activity of esterase in the SML was similar (ANOVA P > 0.05) to UW at both sites (marine zone average EF = 1.1; brackish water zone average EF = 1.5).

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Fig. 2. Bacterial incorporation rate (Vm) of leucine, glucose and acetate in the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro during the sampling period. Error bars represent the standard deviation of three replicates.
Table IV: Bacterial incorporation rate (Vm) of leucine, glucose and acetate and hydrolyze rate (Hm) of aminopeptidase (Leu-AMPase), β-glucosidase (β-GlCase), β-galactosidase (β-GalCase), alkaline phosphatase (APase), esterase and lipase in the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro

<table>
<thead>
<tr>
<th></th>
<th>Marine zone (N1)</th>
<th></th>
<th>Brackish water zone (I6)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SML</td>
<td>UW</td>
<td>SML</td>
</tr>
<tr>
<td>Vm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.6 ± 0.62 (n = 11)</td>
<td>4.4 ± 5.55 (n = 11)</td>
<td>0.2 ± 0.16*</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.2 ± 1.46 (n = 8)</td>
<td>9.5 ± 5.10 (n = 8)</td>
<td>0.3 ± 0.19*</td>
</tr>
<tr>
<td>Acetate</td>
<td>9.5 ± 8.33 (n = 10)</td>
<td>53.8 ± 45.36 (n = 10)</td>
<td>0.3 ± 0.31*</td>
</tr>
<tr>
<td>Hm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-AMPase</td>
<td>1708 ± 2253.1 (n = 8)</td>
<td>1402 ± 1320.5 (n = 9)</td>
<td>1.0 ± 0.29</td>
</tr>
<tr>
<td>β-GlCase</td>
<td>307.2 ± 381.21 (n = 7)</td>
<td>133.1 ± 212.96 (n = 9)</td>
<td>7.3 ± 12.54*</td>
</tr>
<tr>
<td>β-GalCase</td>
<td>18.9 ± 12.12 (n = 10)</td>
<td>11.6 ± 9.40 (n = 10)</td>
<td>3.6 ± 6.18*</td>
</tr>
<tr>
<td>APase</td>
<td>239.1 ± 218.72 (n = 9)</td>
<td>86.7 ± 103.55 (n = 9)</td>
<td>6.6 ± 10.91*</td>
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<tr>
<td>Esterase</td>
<td>532.2 ± 462.67 (n = 8)</td>
<td>535.2 ± 554.00 (n = 9)</td>
<td>1.1 ± 0.66</td>
</tr>
<tr>
<td>Lipase</td>
<td>4.9 ± 2.69 (n = 8)</td>
<td>2.7 ± 1.14 (n = 8)</td>
<td>2.0 ± 1.41*</td>
</tr>
</tbody>
</table>

Average ± standard deviation.
EF, enrichment factor.
*ANOVA, P < 0.01.
**ANOVA, P < 0.05.
N.S., not significant (ANOVA, P > 0.05).

Lipase

Lipase was the enzyme that showed lowest Hm values, varying between 0.5 and 9.7 nmol L⁻¹ h⁻¹ (Table IV; Fig. 4). The highest values were observed in the marine zone, where Hm of lipase was twice as high in the SML than in UW (average EF = 2; ANOVA P < 0.05). In the brackish water zone, the EF of lipase ranged from 0.9 to 3.6 (average 1.5), showing a modest enrichment.

DISCUSSION

Hydrolytic activities in the SML

Consistent and significantly higher hydrolysis rates of the enzymes β-glucosidase, β-galactosidase, APase and lipase were observed in the SML in the marine zone of the estuary. Similar higher hydrolytic rates in the SML environment compared with UW have been observed in other aquatic systems (Müller et al., 1998; Kuznetsova and Lee, 2001; Mudryk and Skorczewski, 2004). Enhanced polymer hydrolysis in the SML is likely due to the accumulation of refractory DOM in the microlayer (Kuznetsova and Lee, 2001). In this work, the relation between hydrolysis rates observed in the SML and a potential DOM enrichment at the air–water interface of the marine zone cannot be confirmed because DOM was not analyzed. Nevertheless, a great variety of organic compounds of natural origin such as proteins, amino acids, carbohydrates, fatty acids, lipids, phenols, as well of anthropogenic origin concentrate at the air–water interface of a range of aquatic systems (Sieburth et al., 1976; Hardy, 1982; Carlucci et al., 1985, 1986, 1991; Williams et al., 1986; Kuznetsova and Lee, 2002; Kuznetsova et al., 2004). Therefore, the concentration of numerous organic and inorganic compounds in the SML can be assumed to be a characteristic feature of this interface.

However, the degree of enrichment is dependent on the trophic status of the water column (Carlson, 1983; Wurl et al., 2011). Carlson (1983) observed that DOM enrichment in the SML diminished with increasing concentrations in the UW. Additionally, Wurl et al. (Wurl et al., 2011) concluded that surfactant enrichment in the SML was higher in waters with lower, than with higher, primary production. The two study sites have different concentrations of organic matter. The average concentration of Chl a in the UW of the marine zone was 1.7 times significantly lower (ANOVA, P < 0.05) than in the brackish water. Furthermore, in the UW of the brackish water zone, DOC is on average, three times significantly
more concentrated than in the marine zone. This different primary productivity and DOC concentration might result in higher enrichments of organic compounds in the SML of the marine than the brackish water zone. Therefore, microbial enzymatic activities in the SML of the estuarine system Ria de Aveiro may essentially reproduce the degree of organic matter enrichment and composition at the air–water interface.

Marine and brackish water zones of the estuary Ria de Aveiro have different amounts and prevalent sources of organic matter. In the marine zone, organic matter is predominantly of autochthonous origin whereas in the brackish water zone there is a mix of allochthonous and autochthonous sources (Almeida et al., 2005). Reflecting these different sources, amounts and composition of organic matter of the estuarine sites studied, the overall enzymatic activity increased at the brackish water zone of the estuary. However, the differences between the SML and UW were higher in the marine zone, particularly the activities of enzymes involved in the hydrolysis of carbohydrates and lipids, and phosphorus acquisition.

In the marine zone, the activity rates of the enzymes involved in the hydrolysis of carbohydrates (β-glucosidase and β-galactosidase) were, on average 7.3 and 3.6 times significantly higher in the SML compared with UW. This enhanced hydrolytic activity might be stimulated by a greater concentration of polysaccharides in the SML environment. The enrichment of polysaccharides in the SML appeared to be a common feature, with EFs ranging from 1.7 to 7.0 for particulate polysaccharides and 3.5 to 12.1 for polysaccharides in the HMW DOM fraction (Gao et al., 2012). In the productive and hydrodynamically stable brackish water, the hydrolytic activity of β-GlCase was only slight and statistically not significantly enriched (EF = 1.3), suggesting low substrate accumulation at the air–water interface of this estuarine zone.

Fig. 3. Hydrolysis rates (Hm) of aminopeptidase (Leu-AMPase), β-glucosidase (β-GlCase) and β-galactosidase (β-GalCase) in the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro during the sampling period. Error bars represent the standard deviation of three replicates.
Lipase activity was the lowest enzymatic activity detected in both marine and brackish water zones, reflecting the universally low concentration of lipids in aquatic systems (Harvey and Mannino, 2001). Despite this low concentration, fatty acid lipids, n-alkanes, and total hydrocarbons are enriched in the SML by factors of 2–5 (Marty et al., 1979). In the present study, we observed a higher lipolytic activity in the SML, particularly in the marine zone of the estuary, suggesting a higher concentration of lipids at the air–water interface, perhaps due to their surface activity and/or high insolubility in water (Hunter, 1997b). An enhanced activity of lipase was previously observed in this estuary and associated with anthropogenic sources of pollution, mainly due to fishery-related activities (Santos et al., 2009). Owing to its proximity to the harbor area, the marine zone is probably exposed to higher levels of pollution, mainly hydrocarbons, which exhibit high accumulation at the air–water interface (Marty and Saliot, 1976; Hardy et al., 1990; Garabetian et al., 1993). A greater concentration of pollutants in the SML of the marine compared with brackish water zone could explain the different enrichments of lipolytic activity observed in the two zones of the estuary.

APase potential activity was always higher in the SML than in UW in both marine and brackish water zones of the estuary. The differences between the two compartments were more pronounced in the marine (6.6 times) than the brackish water zone (2.1 times), suggesting a higher demand for phosphorus in marine zone of the estuary, particularly in the SML compartment. APase is to a large extent a repressive enzyme and is mainly produced during periods of pronounced phosphorus deficiency (Vidal et al., 2003). The marine zone always showed the lowest phosphate concentration (mean value 0.40 μmol L⁻¹) in the estuary (Lopes et al., 2007). Phytoplankton production increases 112 times in the warm season compared with the cold season (Almeida et al., 2002), which might result in an intense seasonal phytoplankton phosphorus demand and consequent increase of APase. The maximum differences between

Fig. 4. Hydrolysis rates (Hm) of alkaline phosphatase (APase), esterase and lipase in the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro during the sampling period. Error bars represent the standard deviation of three replicates.
APase activity in the two compartments were observed during the spring/summer period, suggesting a seasonal decrease of phosphate availability in the SML, promoted perhaps by a higher biomass of phytoneneuston, in comparison with phytoplankton. Unfortunately, phosphate and Chl $a$ concentrations were not determined in the SML, due to the low volume of the samples collected. However, enrichments of phytoneneuston between 1 and $10^2$ have been observed before (Hardy, 1982; Hardy and Apts, 1989) and could occur as well in Ria de Avoiro, particularly in the marine zone. In the brackish water zone, seasonal phytoplankton production variations are weaker (maximum 20.7 times) (Almeida et al., 2002) and consequently the variations of APase activity.

**Monomer utilization in the estuarine SML**

An enhanced hydrolytic activity at the air–water interface might result in a higher concentrations of easy assimilable monomeric compounds. However, in the marine zone of the estuary, bacterioneuston communities showed unexpectedly lower rates of monomer incorporation compared with bacterioplankton. The potential of monomer incorporation by bacterioneuston was only 20 (leucine) and 30 (glucose and acetate) % of that of bacterioplankton at this particular zone of the estuary. This bacterioneuston inability to utilize monomers may be consequence of exposure to higher stress levels in the SML environment or/and unavailability of monomers to uptake.

Reduced potential heterotrophic activities of bacterioneuston compared with bacterioplankton have also been reported by (Dietz et al., 1976) and attributed to a greater stress that bacterial communities are exposed in the SML than in the water column. We recently (Santos et al., 2011) showed that bacterioneuston biomass production (BBP) in the marine zone was on average 10 times lower than bacterioplankton. The low rates of BBP in the SML were associated with the short residence time of bacterial communities as a consequence of the constant mixing between the SML and water column, forced by the hydrodynamic characteristics in this estuarine area. Short residence times prevent bacterial adaptation and metabolic response to the potential organic enrichment of the air–water interface. In contrast, in the brackish water zone water currents are weak and the residence time is longer (Dias et al., 2001). This hydrodynamic stability reduces vertical mixing, promoting stratification and increasing the residence time of the bacterial community at the surface and allowing the exploration of nutrients in the SML (Santos et al., 2011), which might contribute to the similar neutonic and planktonic monomer incorporation rates observed in this specific estuarine zone.

Another possibility is a monomer-reduced availability to bacterioneuston due abiotic adsorption to colloidal DOM. Schuster et al. (Schuster et al., 1998) discovered that dissolved free amino acids (DFAA) readily adsorb to colloidal DOM and particularly to polysaccharides, reducing their availability by two to three orders of magnitude. Considering the gelatinous nature of the SML film, mainly constituted by carbohydrates (Sieburth, 1983; Wurl and Holmes, 2008), the hydrolyzed products might adsorb to the organic matrix reducing their availability to bacteria, which could explain the low rates of monomer incorporation observed in this particular environment. On the other hand, adsorption of DFAA to colloidal matter increases the accessibility of colloidal matter to bacterial degradation (Schuster et al., 1998). The impact of monomer adsorption to colloidal matter and consequent increases of accessibility to microbial enzymatic degradation might be higher in the marine zone, where potentially a higher accumulation of organic matter occurs at the air–water interface as a consequence of the low productivity of the water column.

The present work shows that heterotrophic activities of neutonic and planktonic bacterial communities in the estuarine system Ria de Avoiro show a well-defined pattern of variation. Generally, higher rates of hydrolysis and lower rates of monomer incorporation in the SML compared with UW were observed in the marine zone, whereas, in the brackish water zone, hydrolysis and incorporation rates were similar in the two compartments. This different pattern of variation of microbial activities in the two contrasting estuarine sections might result from different organic and inorganic matter enrichments at the air–water interface, which reflect site-specific characteristics, such as hydrodynamics and organic matter composition.

**CONCLUSION**

In Ria de Avoiro, the differences between hydrolysis and monomer incorporation rates by bacterioneuston and bacterioplankton communities might result from the different amounts and composition of organic matter and the hydrological characteristics of the estuary. In the marine zone, the less productive water column and the strong hydrological mixing promote intense hydrolytic activities and low monomer utilization. In the brackish water zone, a more productive water column and a higher hydrodynamic stability, lead to similar bacterioneuston and bacterioplankton heterotrophic activities. The degree of differentiation between heterotrophic activities of bacterial communities in the SML and UW in estuarine systems is, therefore, a result of the different organic enrichments at the air–water interface and the intensity of exposure to physical processes.
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