Universidade de Aveiro Departamento de Biologia
Ano 2012

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Diversidade filogenética e funcional do bacterioneuston estuarino

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e da Prof. Doutora Isabel da Silva Henriques, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro Programa Alβan - Programa de bolsas de alto nível da União Europeia para a América Latin

Referência da bolsa: E07D403901BR

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Referência da bolsa: SFRH/BD/64057/2009

“Os grandes espíritos têm metas, os outros apenas desejos”

(Washington Irving)

Dedico este trabalho à minha família.

o júri

presidente

Prof. Doutor Casimiro Adrião Pio
professor catedrático do Departamento de Ambiente e Ordenamento da Universidade de Aveiro

Prof. Doutor Artur Luiz da Costa da Silva
professor associado da Universidade Federal do Pará, Brasil

Profa. Doutora Maria Ângela Cunha
professora associada do Departamento de Biologia da Universidade de Aveiro

Profa. Doutora Maria Paula Cruz Schneider
professora associada da Universidade Federal do Pará, Brasil

Prof. Doutor Jorge da Costa Peixoto Alves
investigador auxiliar do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

Prof. Doutor António Carlos Matias Correia
professor catedrático do Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Isabel da Silva Henriques
professora auxiliar convidada do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Agradeço à Universidade de Aveiro, ao CESAM e ao Departamento de Biologia por terem proporcionado as condições logísticas e estruturais para a realização deste trabalho.

Agradeço ao Programa Alβan, Asociación Grupo Santander e Fundação para a Ciência e Tecnologia – FCT – pela concessão da bolsa de doutoramento.

Agradeço ao Professor Doutor António Correia por me ter recebido em seu laboratório e por ter me dado a oportunidade de desenvolver este doutoramento em seu grupo. Também quero agradecer pela orientação e pelas pertinentes decisões em momentos cruciais do desenvolvimento deste trabalho.

Agradeço a minha co-orientadora Doutora Isabel Henriques pela sua incansável tarefa de ensinar. Jamais esquecerei a sorte de lhe ter tido como co-orientadora e seus ensinamentos serão, certamente, perpetuados na minha vida profissional. Obrigada pela amizade estabelecida e pela paciência e persistência na minha formação. Foi fundamental.

Aos meus colegas do laboratório, muitíssimo obrigada pelo excelente ambiente de trabalho que me proporcionaram e por terem aturado algum mau humor em tempos difíceis. Vocês ajudaram-me muito a sentir-me completamente inserida em Portugal ao ponto de muitas vezes eu própria me considerar portuguesa e o vosso Primeiro-ministro como meu também.

Em especial, quero agradecer à Cristina, Susana, Nádia e Eliana por terem dividido comigo momentos mais complicados e me ajudarem a enfrentá-los com palavras de amizade.

À Luísa e Márcia pela amizade, respeito e cumplicidade ao longo desses anos todos e pelo acolhimento em "família", que foi essencial.

Á minha família que deixei do outro lado do atlântico, meus sinceros agradecimentos por terem apoiado este sonho, mesmo que nos tenha custado muitos anos afastados e eu não ter tido a oportunidade de acompanhar o crescimento dos meus sobrinhos João Vítor e Vinícius e minha irmã Ana Carolina.

À minha mãe por ter dedicado a sua vida ao meu crescimento profissional, por me ter transmitido palavras de incentivo e tranquilidade.

Finalmente, ao Luís Miguel agradeço por toda a compreensão, por me ajudar a ultrapassar as dificuldades, pela torcida e pelo amor incondicional.

palavras-chave

Diversidade filogentética; biofilme; micro-camada superficial do mar; estuário; Ria de Aveiro; DGGE; resistência aos antibióticos; genes de resistência aos antibióticos; *Psychrobacter*; psychrofílicos.

resumo

A micro-camada superficial da água (SML) é caracterizada pela ocorrência de grandes quantidades de compostos orgânicos, pela acumulação de contaminantes antropogênicos e é submetida a uma intensa radiação solar, extrema mudança de temperatura e, no caso dos estuários, flutuação de salinidade. Estas propriedades físico-químicas estão, provavelmente, a modular a comunidade bacteriana (bacterioneuston) com propriedades filogenéticas e funcionais específicas. Neste estudo, as abordagens dependentes e independentes do cultivo foram aplicadas para avaliar a estrutura e dinâmica das comunidades bacterioneuston e bacterioplâncton em três localizações geográficas ao longo do estuário da Ria de Aveiro. Além disso, comparámos a diversidade filogenética de grupos específicos (*Aeromonas*, *Pseudomonas* e *Psychrobacter*) presentes em bacterioneuston e bacterioplâncton. Finalmente, as duas comunidades foram comparadas em termos de prevalência e diversidade de bactérias resistentes aos antibióticos e respetivos genes de resistência. Bactérias heterotróficas cultiváveis foram enriquecidas em SML. Eletroforese em gel de gradiente desnaturante (DGGE) permitiu a identificação de filotipos específicos em SML. Além disso, a análise de agrupamento dos perfis de DGGE de ambas as comunidades revelou uma ligeira tendência de agrupamento de acordo com a camada amostrada. As diferenças entre as duas comunidades variaram de acordo com factores espaciais e temporais. Em termos de diversidade filogenética de grupos específicos, não foram identificadas diferenças consistentes entre SML e UW com relação às comunidades de *Aeromonas*. Com relação ao género *Pseudomonas*, uma unidade operacional taxonómica cultivável foi consistentemente hiper-representada nas amostras de SML. Metodologias dependentes e independentes do cultivo revelaram a presença de populações de *Psychrobacter* complexas e muito estáveis em todos os sítios e datas de amostragens, com diferenças significativas entre as comunidades de *Psychrobacter* presentes em SML e UW. Estirpes representativas de prováveis novas espécies também foram cultivadas. Em termos de resistência aos antibióticos, a prevalência de bactérias resistentes em SML foi alta sugerindo selecção pelas condições presentes em SML. É preciso enfatizar que a resistência aos antibióticos foi incomum entre as bactérias estuarinas e os mecanismos de resistência foram, predominantemente, intrínsecos. Pela combinação de abordagens inovadoras dependentes e independentes do cultivo, este estudo forneceu novas e consistentes informações com relação às diferenças em ambas as comunidades bacterianas e em relação a alguns dos fatores que contribuem para a sua formação.

keywords

Phylogenetic diversity; biofilm; sea-surface microlayer; estuary; Ria de Aveiro; DGGE; antibiotic resistance; antibiotic resistance genes; *Psychrobacter*, psychrophilic.

abstract

The water surface microlayer (SML) is characterized by the occurrence of high amounts of organic compounds, the accumulation of anthropogenic contaminants and is subjected to intensive solar radiation, extreme temperature changes and, in the case of estuaries, salinity fluctuations. These particular properties are probably modulating a bacterial community (bacterioneuston) with specific functional and phylogenetic properties.
In this study, culture-dependent and culture-independent approaches were applied to evaluate the structure and dynamics of the bacterioneuston and bacterioplankton communities in three geographic locations along the *Ria de Aveiro* estuary. Additionally, we compared the phylogenetic diversity of specific groups (*Aeromonas*, *Pseudomonas* and *Psychrobacter*) present in bacterioneuston and bacterioplankton. Finally, both communities were compared in terms of prevalence and diversity of antibiotic resistant bacteria and resistance genes.
Cultivable heterotrophic bacteria were enriched in the SML. Denaturing gradient gel electrophoresis (DGGE) allowed identifying SML-specific phylotypes. Also, cluster analysis of DGGE profiles from both UW and SML revealed a slight tendency for grouping according to sampled layer. Differences between both communities varied according to spatial and temporal factors. In terms of phylogenetic diversity of specific groups, consistent differences between SML and UW aeromonads communities were not identified. Regarding *Pseudomonas*, a cultivable operational taxonomic unit was consistently overrepresented in SML samples. Culture-dependent and culture-independent methodologies revealed the presence of complex and very stable *Psychrobacter* populations in all sampling sites and dates, with significant differences between SML and UW *Psychrobacter* communities Strains representing putative new species of *Psychrobacter* were cultivated.
In terms of antibiotic resistance, the prevalence of antibiotic-resistant bacteria was higher in the SML suggesting selection by SML conditions. It has to be emphasized that antibiotic resistance was uncommon among estuarine bacteria and the resistance mechanisms were predominantly intrinsic.
Differences between bacterioneuston and bacterioplankton in *Ria de Aveiro* were detected at different levels: the structure of the total bacterial community, the diversity of specific groups, and in the ability to resist to antibiotics. By the innovative combination of culture-dependent and independent approaches we provide new and consistent information regarding the differentiation of both bacterial communities and some of the factors that contribute for their shaping.

List of original publications

This thesis includes results published in the articles listed below. Additionally, some unpublished results are presented.

Azevedo JSN, Ramos I, Araújo S, Oliveira CS, Correia A, Henriques I (2012) Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies. *Antonie van Leeuwenhoek*, **101**, 819 – 835. – Results presented in **Chapter 3**.

Azevedo JSN, Correia A, Henriques I (2012) Molecular analysis of the diversity of genus *Psychrobacter* present within a temperate estuary. *FEMS Microbiology Ecology* (in revision). – Results presented in **Chapter 4**.

Azevedo JSN, Araújo S, Oliveira CS, Correia A, Henriques I (2012) Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine environment. *Microbial Drug Resistance* (accepted for publication). – Results presented in **Chapter 5**.

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1. Introduction

1.1. The water surface microlayer (SML)

The water surface microlayer (SML) represents the boundary interface between hydrosphere and atmosphere defined as roughly the uppermost 1 to 1,000 µm of the water surface (Liss et al., 1997). This layer is represented by a thin biogenic film, naturally occurring in marine, estuarine and freshwater water bodies and covering almost 71% of the world's surface (Hardy, 1982; Hale & Mitchell, 1997).

1.1.1 Dimension and physicochemical properties of SML

Historically, the estimation of the thickness of the SML varies widely, due to different sampling protocols (Zhang et al., 1998, 2003; Zhengbin et al., 1998). Nevertheless, it is generally accepted that SML does not exceed 1,000 µm in depth (Hunter, 1980; Broecker & Peng, 1982).

The early descriptions of this layer resulted on a classical model of a stratified structure comprising an upper lipid layer containing "dry" active-surfactant fatty-acids, long-chain alcohols and lipids (typically considered to be of very low solubility) covering the subsequent "wet" surfactant protein-polysaccharides layer. In the next sub-layers of SML many aquatic organisms can be found (Figure 1.1a) (Hardy, 1982; Hermansson, 1990).

Collectively aquatic organisms within the microlayer are known as the neuston, and the community of bacteria present within this neuston layer was named the bacterioneuston (Naumann, 1917). According to the classical model of stratified layers, immediately below the bacterioneuston compartment, deeper layers of phytoneuston and zooneuston can be found (Zuev et al., 2001).

However, recent evidences indicate that instead of a stratified microlayer, the SML is represented by a hydrated gelatinous microlayer film comprising macromolecules and colloids that are mainly produced from dissolved organic matter (DOM) and particulate organic matter (POM). A microbial community is attached to this gelatinous film (Figure 1.1b) (Walczak & Donderski, 2004; Wurl & Holmes, 2008; Cunliffe et al., 2009b).

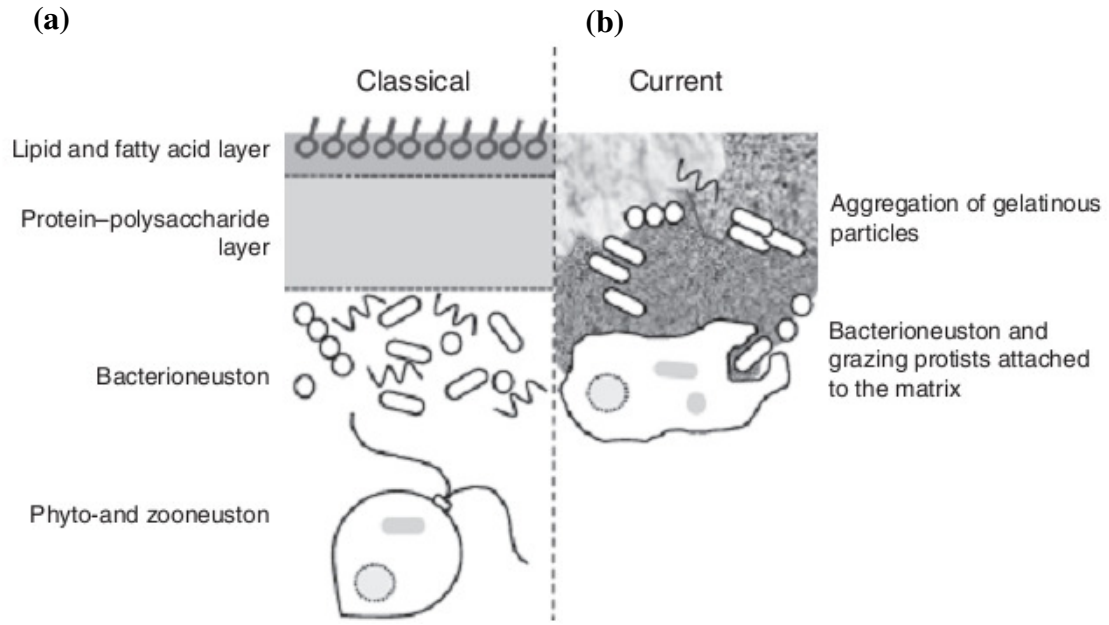

Figure 1.1 Schematic model of the structure of the SML. **(a)** The classical model of a stratified structure showing two layered surface microlayer: upper layer containing "dry" surfactant lipids covering the underlying "wet" layer composed of protein-polysacharides. **(b)** The current model of hydrated gelatinous microlayer film enriched with TEP and associated microbial life. Picture was extracted from Cunliffe et al, 2011.

This proposal was first advanced by Sieburth (1983) and was established based on many observations made over several years, including indications that lipids were not present in sufficient concentrations to justify the classical model (Williams et al., 1986).

Additionally, microbial cells collected from SML of the Sargasso Sea exhibited amylolytic activity and lacked lipolytic and proteolytic activities, leading to the conclusion that the surface films originated mainly from released carbohydrates rather than lipids (Sieburth & Conover, 1965). Thus, Sieburth (1983) hypothesized that the distinct slick reported directly above the bloom was formed by a complex structure of polysaccharides and proteins. His proposal was in congruency with earlier studies by Baier et al. (1974) which also suggested the dominance of polysaccharides and proteins in the composition of the SML.

One of the most ubiquitous groups of gel particles present in the marine environment is known as transparent exopolymer particles (TEP) (Verdugo et al., 2004). TEP are

generally formed in the SML by the coagulation of biogenic polysaccharides and proteins, mainly produced by phytoplankton, reaching the SML compartment and acting as a binding matrix that maintain SML aggregates together (Verdugo et al., 2004).

Experiments evaluating the capacity of seawater containing TEP precursors in forming TEP *de novo* also showed that TEP were able to aggregate latex spheres, which were used to mimic microbial cells inhabiting the binding matrix (Azetsu-Scott & Passow, 2004). These results corroborate the hypothesis that a microbial community can be found intimately attached to the gelatinous film. These findings also contradict the classical model of the SML in which the bacterial community is immediately below the "dry" and "wet" surfactant layers, consequently not mixed within the biofilm.

Evidences based on fluorescent *in situ* hybridization analysis with probes targeting *Bacteria*, *Betaproteobacteria* and *Gammaproteobacteria* also revealed that TEP in SML may facilitate the aggregation of bacterial cells in this layer rather than in underlying water (UW), in which bacterial cells seem to be more dispersed (Cunliffe & Murrel, 2009).

Wurl and Holmes (2008) have identified TEP as being attached to particulate-forming-aggregates. Moreover, they compared the concentration of TEP from SML and UW in oceanic and estuarine water from Southeast Asia. They showed that the TEP enrichment factor was higher in SML, especially in estuarine water (Wurl & Holmes, 2008). Similarly, significant TEP enrichment in SML was identified in experimental mesocosms using Norwegian fjord waters to stimulate phytoplankton blooms (Cunliffe et al., 2009c).

Enrichment factor (EF) is basically defined as the concentration of a specific component in the SML divided by its concentration in UW. This measurement is a standard practice in SML research to specify the microlayer depth. As previously stated, SML depth is still under discussion, probably because there is currently no consensus as to the most appropriate strategy for sampling the surface microlayer, constraining comparative analysis.

Despite of that, the enrichment of several compounds often detected in the SML arises mainly from the fact that it is the first layer of water that receives wet and dry atmospheric deposition (Hardy, 1982). Atmospheric deposition of matter has been reported to be a very important input for the enrichment of SML (Wotton & Preston, 2005). The high amounts of compounds from atmospheric deposition and also those from biogenic sources such as protein, lipids and organic surfactants increases the SML film stability, which,

consequently, promotes an enrichment in particles, organisms and dissolved material (Zuev et al., 2001).

Enrichment also occurs by bursting bubbles containing microorganisms, including viruses, proteinaceous material and gel particles (Kuznetsova et al., 2005), going upward to the SML via positive buoyancy (Azetsu-Scott & Passow, 2004).

Due to its distinct chemical composition, this interface also acts as both a sink and a source of anthropogenic persistent organic pollutants (POPs), including pesticides, polychlorinated bi-phenyls (PCBs), organotin compounds, polycyclic aromatic hydrocarbons (PAH) and heavy metals (Agogué et al., 2004; Wurl & Obbard, 2005; Obernosterer et al., 2007; Cuong et al., 2008; Wurl & Holmes, 2008; Wurl et al., 2009). In fact, many studies have quantified a wide spectrum of chemical contaminants that preferably accumulate in the SML compared to UW in different aquatic systems (this topic was deeply reviewed by Wurl & Obbard, 2004). Those studies have shown that the enrichment factor of SML samples from coastal and estuarine waters was higher when compared to the open ocean (William & Robertson, 1973; Fowler 1990; Wurl & Obbard, 2004). This stronger enrichment in coastal and estuarine SML can be mainly attributed to shipping activities and terrestrially derived material from wastewater discharges, agricultural and industrial run-off and atmospheric deposition of combustion residues (Wurl & Obbard, 2004).

POPs represent a wide range of recalcitrant xenobiotic chemicals with known toxicological effects in the marine environment. The enrichment of POPs in the SML represents a potential threat to marine biodiversity and, in general, SML concentrations of PCBs, dichlorodiphenylethanes (DDT) and PAHs could be higher by factors of up to 10, 40 and 113 respectively compared to underlying waters (William & Robertson; Cincinelli et al., 2001). Also it may have strong economical impacts by affecting fishery activities and aquacultures. In fact, undesirable effects have already been reported on endocrine systems of a sort of aquatic organisms, including mammals (Tanabe, 2002; Bosveld & van den Berg, 2002) and fish larvae (Cross et al., 1987), mainly related to the accumulation of pesticides and PCBs.

Another potentially toxic group of pollutants that preferably accumulates within the SML are organotins (Gucinski, 1986), namely tributyltin (TBT), which is a common contaminant of marine and freshwater ecosystems due to its use as an antifouling agent in

boat paints. As a result of its widespread contamination, chronic and lethal effects on aquatic organisms, such as algae, zooplankton, molluscs and the larval stage of some fishes are well known (Leung et al., 2006; Choi et al., 2009; Liu et al., 2011). TBT or even products from its degradation have been identified as occurring preferably in SML from semi-enclosed water bodies, such as estuaries and marinas, as well as in shipping channels and harbours rather than in rivers and offshore (Arambarri et al., 2003; Nogueira et al., 2003).

Higher concentrations of PAHs have also been detected in SML (Anikiyev & Urbanovich, 1989), particularly at sampling locations where human coastal activities are intense. Therefore, these areas are highly influenced by discharges from shipping, by the size of the port and its intensity of shipping traffic and also by the limitation in water exchange, which collectively can strongly concentrate these contaminants in the microlayer (Zeng & Vista, 1997; Cincinelli et al., 2001). Pollution by PAHs is particularly hazardous to all compartments of the environment because they are highly toxic, carcinogenic and teratogenic compounds (Burton et al., 2006). PAHs released into the environment are mainly from anthropogenic sources, through contamination by crude oils, coal, coal tar or pyrolytic origin.

Concerning heavy metals, their concentrations normally decrease with distance from coastline owing the fact that heavy metals are often associated to organic ligands, which are more likely to occur due to anthropogenic discharges (Cross et al., 1987; Hardy & Cleary, 1992). As dissolved and particulated organic matters are frequently enriched in the SML compartment, this interface has been recognized as a "hot spot" for the enrichment of heavy metals in aquatic ecosystems (Hunter & Liss, 1981; Hardy et al., 1985; Cuong et al., 2008). In fact, Cuong et al. (2008) have found higher concentrations of arsenic, copper and nickel especially in the particulate fraction of the SML compartment from Singapore's coastal waters. Moreover, an enrichment factor of 1.33 of dissolved organic matter (DOM) in the SML compared to UW from Jiulong estuary (China) has led to higher concentrations of Cu and Cd in the SML (Hong & Lin, 1990).

Many heavy metals are crucial to the metabolism of various aquatic organisms. However they become potentially toxic to aquatic life if their concentration reaches the threshold of bio-availability (Blackmore, 1998). Sources of heavy metals in aquatic environments include atmospheric deposition, riverine inputs, wastewater discharges and

re-suspension of contaminated bottom sediments (Mart et al., 1982; Poikāne et al., 2005). In summary, regardless of the aquatic ecosystem that is being considered, many studies have indicated that SML, compared to the underlying water column, is a potential site of enrichment of heavy metals and a diversity of other pollutants, thus having a pivotal role in the distribution of these contaminants globally, with relevant implications regarding aquatic ecotoxicology, including mortality, abnormal development of a wide range of organisms (mainly at first stages of life, including larvae) and depression of grow rates (Cross et al, 1987).

SML is also subjected to seasonal and diurnal fluctuations with greater extremes in temperature, salinity and solar radiation when compared to UW (Hardy, 1982). In fact, owing to strong direct solar radiation affecting the SML, this interface often become on average 0.1 to1.4ºC warmer than the troposphere (Zuev et al., 2001). Additionally, solar radiation promotes photolysis of DOM resulting on reactive radicals such as singlet oxygen and hydroxyl radicals (Miller, 1994; Zepp et al., 1995; Santos et al., 2011a) thus making this microlayer into a hostile environment for living organisms.

1.1.2 Biological properties

Organisms present in the SML may have developed life strategies to survive in this inhospitable habitat exposed to intense solar radiation, strong temperature and/or salinity gradients, toxic organic substances, and harmful concentrations of heavy metals (Maki, 1993). Still, a wide spectrum of organisms can be observed at the extremophile microhabitat found in the SML, generally in higher abundances than in UW (Hardy, 1982). These organisms are named neuston (Naumann, 1917) and include phytoneuston, bacterioneuston and zooneuston.

Phytoneuston has a pivotal role in autotrophic production in the SML, owing the photosynthetic activity of a great variety and density of microalga occurring in this interface (Hardy, 1973). Phytoneuston composition has a particular significance on air–sea gas fluxes and also supports higher trophic levels. In fact survival rates of numerous types of invertebrate larvae depend on microalga availability in the SML (Hardy, 1982). Phytoneuston populations are frequently dominated by Chrysophyta, Chlorophyta, Euglenophyta and neustonic diatoms (Hardy, 1971) and studies based on phytoneuston

taxonomical composition often pointed differences between SML and UW mainly by the dominance of *Cercozoa* and *Ciliophora* in the SML (Williams et al., 1986; Hardy & Apts, 1984, Cunliffe & Murrel, 2010). Additionally, surveys based on pigmented-signatures between phytoneuston and phytoplankton communities in a coastal lagoon of Baja California revealed differences in both communities with a greater concentration of two pigments in the SML, however not detected in UW, which was enriched with other types of pigments depleted in the SML (Montes-Hugo & Alvarez-Borrego, 2007).

The secondary productivity in the SML depends upon the zooneuston, mostly represented by small and large metazoan and eggs, larvae and small fishes including some with high commercial value (Barrtlett & Haedrich, 1968; Zaitsev, 1971). Zooneuston has a critical role in the food aquatic network being consumed by higher trophic levels and also being responsible for the consumption of phyto- and bacterioneuston (Hardy, 1971). It has been demonstrated that SML contains an abundance and diversity of bacteria, the so called bacterioneuston (Sieburth et al., 1976; Münster et al., 1998). Some estimates commonly state that the bacterioneuston is 10^2 to 10^3 times more abundant than the bacterioplankton at the same sampling sites (Bezdek & Calucci, 1972). Enrichment of dissolved organic matter often found in the SML has been suggested as the main factor driving the enrichment of bacteria (Sieburth et al., 1976). The bacterial abundance present in the SML could therefore create different protist niches supporting the surface microlayer-specific protist communities already observed (Cunliffe & Murrel, 2010).

1.1.3 Studying the SML: sampling methodologies

One of the challenges when analyzing the SML is to choose the adequate method to collect the surface pellicle in the specific conditions of the system under study. Several factors such as wind speed, biological and physicochemical properties and the volume of sample required are important in selecting the sampling strategy.

Regarding to the bacterioneuston field, four sampling methods are often applied: the metal screen – MS (Garrett, 1965), the rotating drum – RD (Harvey, 1966), the glass plate – GP (Harvey & Burzell, 1972) and hydrophilic/hydrophobic nucleopore membranes (Crow et al., 1975; Kjelleberg et al., 1979) (Figure 1.2; Table 1.1).

The MS consists of a stainless steel screen which is oriented horizontally and lowered through the microlayer into the subsurface water before being slowly withdrawn in the same way (Garrett, 1965).

Rotating drum sampler uses a smooth, gyratory cylinder which surface is readily wet by water. A large neoprene blade is pressed tightly into the surface of the cylinder to remove continuously the film and water. Rotation is accomplished by a storage battery operated synchronous stepping motor with reducing gear (Harvey, 1966).

Unlike MS, the GP device is introduced vertically, lowered through the microlayer into the subsurface water and slowly raised back out. Water samples are drained using a wiper blade and then, the adhered sample is stored into a sterile bottle (Harvey & Burzell, 1972).

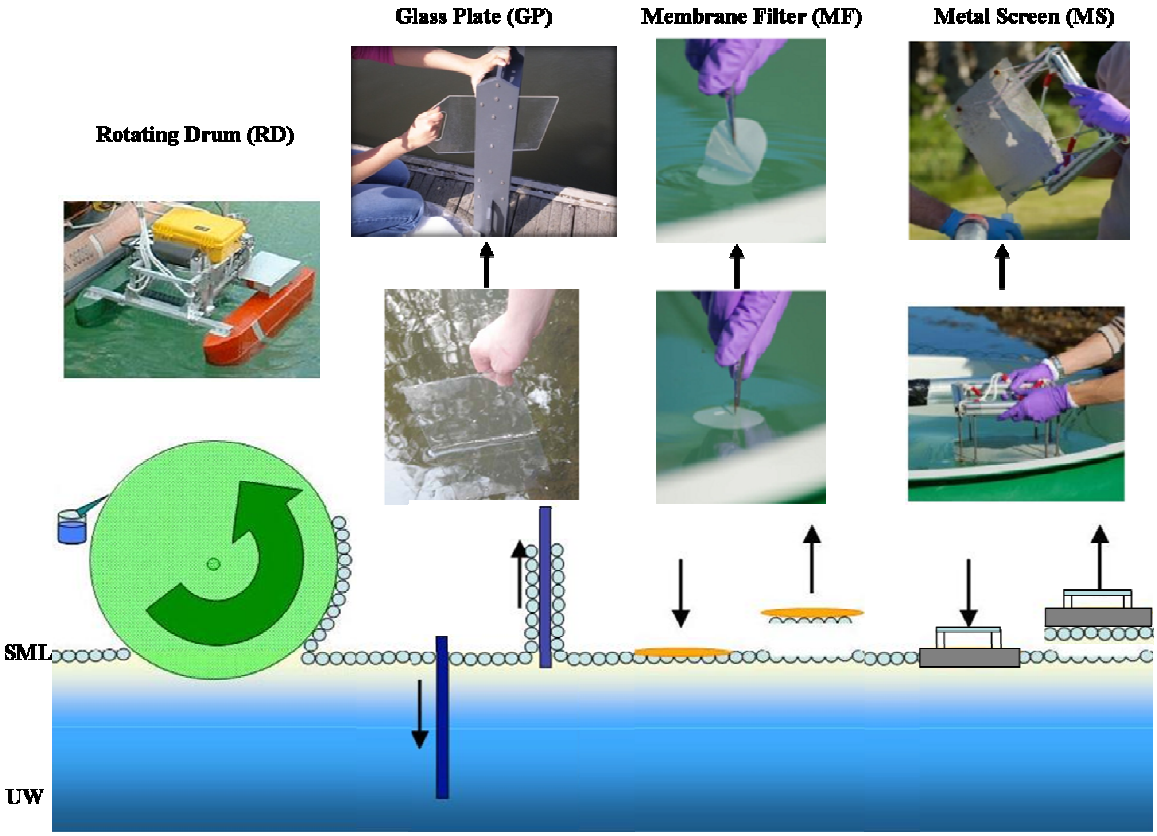

Figure 1.2 SML sampling devices often used: Rotating Drum – RD – (Harvey, 1966); Glass Plate – GP – (Harvey & Burzell, 1972); Metal Screen – MS – (Garrett, 1965); Membrane filters – MF : polycarbonate membrane (Crow et al., 1975) and polytetrafluoroethylene (Teflon) (Kjelleberg et al., 1979).

Adhesion sampling is also possible by using sterile hydrophilic (polycarbonate) or hydrophobic (teflon) membrane filters placed directly onto the water surface and then lifted up with the surface microlayer attached and placed into storage vessels for processing (Crow et al., 1975; Kjelleberg et al., 1979).

Although there are controversial opinions regarding the sampling bias associated to the membrane properties, the potential of contamination with water column is considerably minor than the one expected when using MS or GP devices. On the other hand, the sample volume obtained is smaller when using membranes. Moreover, many authors have suggested that bacterioneuston tend to be concentrated in the uppermost 1 μm (Norkrans, 1980; Hermansson, 1990) and thus, membrane filters would be more appropriated for sampling.

Field studies comparing the efficiency of different sampling devices on given SML microbial community are relatively scarce. Agogué et al. (2004) compared three methods for sampling SML of the Mediterranean Sea to estimate chlorophyll concentrations, bacterial production, bacterial cell numbers (total and cultivable), viruses, flagellates and ciliates. There was no discrepancy in those analyses using either MS or GP, though the thickness of the SML sampled by GP device is thinner than the one sampled by MS (Table 1.1). Thus, GP should be preferable to collect SML, since the thinner is the layer collected, more representative will be the sampled community. On the other hand, MS gives a level of contamination much lower than GP due to it being oriented horizontally during sampling events. Additionally, the MS method often obtains higher volumes than the GP, what might be an advantage for studies that require higher sample volumes (Agogué et al., 2004). Regarding SML sampling with membranes, the same authors have suggested that selective adherence of cells to Teflon and polycarbonate membranes resulted in biased numbers of bacterial cells (total and cultivable).

Recently, Cunliffe and co-workers (2009a) re-evaluated both membrane types as well as MS and GP methods specifically for molecular analysis of SML bacterial communities. Denaturing gradient gel electrophoresis (DGGE) revealed that bacterial community profiles from SML samples collected using both membrane types were 78% similar to those obtained from samples collected using the MS and GP devices. Additionally, profiles from UW samples (sampling depth 0.4m below surface) collected with both types of membranes and MS and GP devices grouped together. From this it was concluded that the

use of membranes does not select for a different microbial community (Cunliffe et al., 2009a).

Still, the most appropriate sampling method for sampling bacterioneuston community remains undefined. Choosing the SML sampling method is an essential step and the choice must take into account the main goal of the research project. For instance, for studies considering the influence of tidal regimens the time of sampling must be restricted to a minimum to avoid temporal variability. In such cases, a method able to collect high volumes in a short period would be more appropriate.

The structure, thickness and composition of SML varies in different ecosystems (Wurl et al., 2011) and such variations might be driven by physical and atmospheric conditions, such as input of wet/dry material, rainfall events, wind speed and tidal cycles, as well.

Specifically, wind speed factors can greatly contribute for SML-forming and its dynamics (Hale & Mitchell, 1997). Several studies have assumed that wind speeds of < 3-4 m s^{-1} are the limit for SML sampling due to its surface-tension stability (Sieburth et al., 1976; Williams et al., 1986; Agogué et al., 2004; Reinthaler et al., 2008), however, Wurl et al. (2009; 2011) have demonstrated that enrichment of surface-active compounds in the SML can persist at wind speeds greater than 6 m s^{-1}.

Nonetheless, in general, indications for collecting SML should avoid conditions that may disturb the stability of the SML-biofilm, especially high wind speed and rainfall events during sampling procedures. For comparative studies of temporal and spatial effects on the dynamics of SML, sampling must be conducted under the same conditions of light regimens and tidal cycle for instance, besides low wind speed conditions.

Table 1.1 Summary of the advantages and disadvantages of the four most commonly used surface microlayer sampling methods

SML Sampling devices and depth (µm)		Sample collected	Advantages	Disadvantages	References
MS	150-400	Microbes, lipids and fatty acids	Collects a relatively large sample volume, which facilitates more extensive analysis	As the sampling depth is deep, the sample collected can be a mixture of both SML and UW. Difficulty in sterilize between sampling events.	Carlson, 1982; Agogué et al., 2004; 2005a,b; Joux et al., 2006; Obernosterer et al., 2008; Cunliffe et al., 2009a.
RD	60-100	Microbes and organic matter	Collects exclusively SML	Expensive and difficult to standardize operation	Harvey, 1966
GP	20-100	Chemical compounds and microbes	Same as mesh screen (MS). Additionally, collects the closest biological composition to the original in the SML.	Same as with MS	Agogué et al., 2004; 2005a,b; Cunliffe et al., 2009a.; Wurl et al., 2011.
PC	4-40	Microbes and organic matter	Collects exclusively SML; cheap and easily available	Collects a relatively small sample volume; bias associated to bacterial counts; difficult to use in high wind speed	Agogué et al., 2004; Franklin et al., 2005; Cunliffe et al., 2009a.
PTFE	20-50	Microbes and organic matter			

Metal Screen – MS – (Garrett, 1965); Rotating Drum – RD – (Harvey, 1966); Glass Plate – GP – (Harvey & Burzell, 1972);

Polycarbonate membrane – PC – (Crow et al., 1975); Polytetrafluoroethylene membrane (Teflon) – PTFE – (Kjelleberg et al., 1979).

1.2. Bacterial community inhabiting the SML

As described above, the SML constitutes a unique physical, chemical and biological environment, different from deeper water, in which preferential deposition of organic matter, heavy metals and several pollutants occur. Also, this layer is subjected to strong temperature and/or salinity gradients (Liss & Duce, 1997) and to intense solar radiation (Agogué et al., 2005b). Thus, this environment gathers strong selective pressures affecting organisms associated to this interface.

Hence, organisms present in the SML must have developed life strategies to survive in this microhabitat, often considered as an extreme environment. Consequently several studies suggested the presence of novel and unusual taxa in SML (Maki, 1993; Joux et al., 1999; Maki, 2002).

Microorganisms in the SML are subjected to a combination of both favorable and detrimental factors. For instance, the enrichment of organic material as a favorable factor may fuel the bacterioneuston community. In fact, different reports suggest that bacterial concentration in SML exceeds that of UW by orders of magnitude (Sieburth et al.1976; Hardy, 1982). Despite their abundance and expected widespread distribution, whether this unusual habitat determines a specific bacterial community remains unclear. The bacterial communities thriving at the SML are still poorly characterized in terms of phylogenetic composition and functional traits.

1.2.1 Phylogenetic diversity

Studies on the microbial ecology in the SML have reported conflicting results. Franklin and co-workers (2005) have chosen the membrane method for sampling the SML from the United Kingdom North Sea coast. Analysis of *Bacteria 16S* rRNA gene libraries constructed from DNA obtained from SML and UW revealed that the bacterioneuston was distinct from bacterioplankton, displaying significantly lower bacterial diversity (with only 9 operational taxonomic units - OTUs) being mainly represented by two genera: *Vibrio spp.* (68% of clones) and *Pseudoalteromonas spp.* (21% of clones). In contrast, the bacterioplankton library showed 46 OTUs. The dominance of these genera was further confirmed by using gene probes specific for these two groups. Hybridization against 1,000

clones from bacterioneuston and bacterioplankton libraries revealed that 57% of clones from the bacterioneuston library hybridized to a *Vibrio*-specific *16S* rRNA gene probe and 32% hybridized to a *Pseudoalteromonas*-specific *16S* rRNA gene probe. In contrast, the bacterioplankton library resulted in only 13% and 8% of *16S* rRNA gene clones hybridizing to the *Vibrio* and *Pseudoalteromonas* probes respectively.

Agogué et al. (2005a) compared the bacterioneuston and bacterioplankton structure by using culture and genetic fingerprinting methods. Samples were obtained using the MS and GP samplers from two sampling sites: the oligotrophic Bay of Banyuls-sur-Mer, France, and a moderately eutrophic area of Olympic Harbour in Barcelona, Spain. Proteobacteria were consistently more abundant in the collection from the pristine environment whereas Gram-positive bacteria were more abundant in the polluted site, especially in SML samples, where *Actinobacteria* were prevalent. Based on single-strand conformation polymorphism profiles (SSCP) of *Bacteria 16S* rRNA genes, only a few additional peaks were found in SML samples from the eutrophic sampling point, suggesting the occurrence of minor differences between SML and UW bacterial communities. Besides that, authors reported no consistent difference between the bacterioneuston and the bacterioplankton at either site.

Latter, the bacterial communities in the SML and UW at two sites along a small tidal estuary, located on the North Sea coast of the United Kingdom, were compared using culture-independent molecular-based approaches, namely DGGE and *16S* rRNA gene libraries (Cunliffe et al., 2008). DGGE profiles revealed that microbial community composition remained relatively similar (similarity 88%) in UW at both sampling sites and many of the dominant *16S* rRNA gene amplicons, present as intense bands in UW DGGE profiles, were also present in DGGE profiles from the SML, showing that comparatively to SML, those intense DGGE bands represent abundant taxa in the UW community. On the other hand, DGGE profiles from SML samples showed *16S* rRNA amplicons (DGGE bands) not detected in UW samples or being only dominant in SML samples, thus indicating SML-specific microbial populations. Those DGGE bands were excised and sequenced. Sequences were similar (91 - 100%) to *16S* rRNA gene sequences from members of *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteriodetes* retrieved from a large variety of marine and estuarine habitats. Additionally, dominant amplicons found in SML were affiliated to *Glaciecola* spp. and *Alteromonas*, which are phylogenetic relatives

of the genus *Pseudoalteromonas*, already reported as a dominant component of the bacterioneuston from the coastal North Sea (Franklin et al., 2005). Regarding the *16S* rRNA gene libraries, although libraries from SML and UW samples shared several OTUs, most OTUs in both libraries were unique to that library, revealing that SML-specific or UW-specific bacterial populations were sampled.

In another study, the bacterioneuston DGGE profiles from two sampling sites, on opposite sides of the Hawaiian Island of Oahu in the Pacific Ocean, were more similar to each other than to the corresponding bacterioplankton (Cunliffe et al., 2009a).

Similar results were obtained in a mesocosm-based study (Cunliffe et al., 2009c). Both communities were profiled using DGGE and distinct profiles were obtained for bacterioneuston and bacterioplankton. SML-specific DGGE bands were sequenced and phylogenetically affiliated to two families: the *Flavobacteriaceae* and the *Alteromonadaceae* (Cunliffe et al., 2009c).

Only a few studies were conducted aiming to compare the bacterial communities inhabiting the SML and UW in freshwater systems. The existence of a distinct bacterioneuston community was investigated in an alpine lake during two consecutive ice-free seasons (Hörtnagl et al., 2010). They verified that SML and UW communities were in general similar and dominated by *Actinobacteria* and *Betaproteobacteria*. However, they identified a few specific bacterial members of *Betaproteobacteria* as being efficient colonizers of the SML from alpine lakes.

More recently, the effect of meteorological conditions (namely wind speed and solar radiation) on bacterioneuston and bacterioplankton community structures were examined taking into consideration non-attached and particle-attached bacterial assemblages (Stolle et al., 2011). Authors have concluded that non-attached communities of the SML and UW were very similar and differences between them were only observed with low wind speed and high radiation levels. In contrast, the difference in the particle-attached community structure between the bacterioneuston and the bacterioplankton were more pronounced, suggesting that particulate organic material accumulation seems to differentiate the community structure of bacterioneuston from that of bacterioplankton, rather than meteorological conditions. Additionally, SML-specific SSCP bands from *16S* rRNA fingerprints of the particle-attached and non-attached fractions were sequenced and were

phylogenetically related to *Cyanobacteria*, *Bacteroidetes*, and *Alpha-*, *Beta-*, and *Gammaproteobacteria* originally found in diverse habitats.

Most of the studies designed to compare the structure of the bacterial communities inhabiting the SML and UW have focused on small collections of bacterial isolates or, when culture-independent methods were used, on the comparison of the composition of the communities at high taxonomic levels. Studies focusing on differences between layers in terms of occurrence and diversity of specific bacterial groups were not conducted before. During the present study efforts were made to detect differences at the genus level. For this the genus *Psychrobacter* was selected since it was one of the most frequently retrieved genera in the estuary under study (**Chapter 4**). For this reason a summary of the characteristics of the members of the genus *Psychrobacter* is presented below.

1.2.1.1 ***Psychrobacter***

The genus *Psychrobacter* at the moment of writing comprises 34 validly published species names (http://www.bacterio.cict.fr/p/psychrobacter.html) and most of them were retrieved from frozen habitats such as glacial ice, permafrost and sea-ice samples. *Psychrobacter immobilis* is the type species for this genus, which was originally described by Juni and Heym (1986) to accommodate Gram-negative, cold-adapted, oxidase-positive, non-motile and non-pigmented coccobacilli with strictly oxidative metabolism. The current taxonomic classification places the genus *Psychrobacter* as follows: domain Bacteria; phylum Proteobacteria; class Gammaproteobacteria; order Pseudomonadales; family Moraxellaceae (http://www.ncbi.nlm.nih.gov/taxonomy.) The family Moraxellaceae also includes the genera *Enhydrobacter*, *Acinetobacter*, *Moraxella*, *Alkanindiges* and *Perlucidibaca* (Staley et al., 1987; Rossau et al., 1991; Enright et al., 1994; Bogan et al., 2003; Song et al., 2008).

Psychrobacter members are known for their psychrotolerance and halotolerance (Vishnivetskaya et al., 2000). Microorganisms adapted to cold conditions can be classified according to their optimum growth temperature: psychrophilic for those with optimum growth temperature bellow 15°C; and psychrotrophic or psychrotolerant for those able to grow under 15°C, though higher temperatures ranging from 15°C to 50°C are preferable (Morita, 1975; Cava, et al., 2009). All *Psychrobacter* species described so far grow well

between 4 to 20°C (Bowman, 2006). Only five species of this genus can grow up to 37ºC (Juni & Heym, 1986; Bowman et al. 1996; Kämpfer et al. 2002; Vela et al. 2003; Wirth et al. 2012).

Several new *Psychrobacter* species have been described in the last few years, mostly from the increasing exploration of marine and polar ecosystems.

There are five main characteristics useful for differentiating *Psychrobacter* from its closest relatives *Moraxella* and *Acinetobacter*. In comparison to *Psychrobacter*, *Moraxella* and *Acinetobacter* are mesophilic, unable to grow at 4°C and are not halotolerant or halophilic. In general, neither of these genera occurs in marine environments, as *Psychrobacter* does. Although, *Psychrobacter* and *Moraxella* are oxidade positive and *Acinetobacter* is not, the genus *Moraxella* is generally isolated from animal tissues and fluids requiring fastidious growth media (Rossau et al., 1991).

Phenotipically, bacteria belonging to *Psychrobacter* genus generally form cream or off-white, smooth, circular, convex colonies with a smooth margin and a buttery consistency. Occasionally, due to accumulated cytochrome proteins, the colonies of a few species become pale pink (Bowman et al., 1997).

Psychrobacter species are Gram-negative, although the cells can sporadically retain the crystal violet dye giving the perception that they are Gram-positive. Despite of that, cells can be lysed easily in the presence of detergents or strong alkaline solutions unlike most Gram-positive cells. Additionally, cells are classified as nonmotile, with no resting bodies like spores or cysts (Juni & Heym, 1986).

Salinity requirement and tolerance of 1- 6% NaCl can be observed for all *Psychrobacter* species, due to their halotolerance (Bowman et al., 1996). Only two *Psychrobacter* species can tolerate up to 15% NaCl (Romanenko et al., 2002) and halotolerance is one of the criteria adopted for differentiating *Psychrobacter* species. Additionally, a minority of species require sodium to start growing (Romanenko et al., 2002; Bozal et al., 2003), although most species can grow in the absence of salt (Juni & Heym, 1986; Kämpfer et al., 2002; Yoon et al., 2003; Yumoto et al., 2003). In summary, *Psychrobacter* isolates are strongly stimulated to grow in growth media supplemented with 0.5 – 1.0M of NaCl (Bowman et al., 1997).

Generally, environmental isolates have capacity to grow on various organic rich media commonly used in the laboratory and clinical specimens, preferentially, grow on Brain

Heart Infusion (BHI) or blood/serum agar (Bowman et al., 1996; Vela et al. 2003; Wirth et al., 2012). Despite of that, the majority of *Psychrobacter* species described so far are unable to metabolize complex substrates, such as polysaccharides and carbohydrates (Bowman et al., 2006). On the other hand, proteolytic activity and acid production from some carbohydrates have already been reported (Maruyama et al., 2000; Denner et al., 2001; Romanenko et al., 2002).

Psychrobacter species often produce lipases that can breakdown a few substrates such as uric acid that, normally, would not be catabolized by most Gram-negative bacteria. In fact, *Psychrobacter* isolates were already reported as being able to hydrolyse uric acid and also grow on uric acid and its metabolites as sole carbon and energy sources (Bowman et al., 1996).

Psychrobacter strains are taxonomically characterized by a polyphasic approach, including *16S* rRNA gene sequencing, DNA–DNA hybridization, fatty acid analysis, morphological and biochemical analyses. Among these methods, the characterization of fatty acid content is determinant for the identification of the genus and also to distinguish species.

Major fatty acids in *Psychrobacter* include the monounsaturated lipids, namely, palmitic acid, heptadecenoic acid and oleic acid. Their predominance is due to their low melting point that helps maintain cytoplasmic membrane permeability at low temperatures (Russell, 2003). The production of wax esters may be also related to cold-adaptive microorganisms and vary significantly between strains and species (Russell & Volkman, 1980).

Only three representative strains of the genus *Psychrobacter* had their genomes completely sequenced so far (http://www.ncbi.nlm.nih.gov/genome/?term=psychrobacter). Those strains belong to two validly published species, *P. cryohalolentis* (GenBank accession number CP000323) and *P. articus* 273-4 (Ayala-del-Río et al., 2010) and the third genome is from a novel *Psychrobacter* member not yet published as a valid species (Kim et al., 2012).

Sequenced genomes vary from 2.65 to 3.51Mb and contained 2,221 to 2,713 open reading frames. The G+C content ranged from 42.2% to 43.4%. Approximately 80% of nucleotides were predicted as protein-coding regions.

Studies regarding the genus *Psychrobacter* are mainly addressed to unravel survival and stress conditions in response to low-temperature (Amato & Christner, 2009; Song et al., 2012; Novototskaya-Vlasova et al., 2012). Proteomic studies (Bakermans et al., 2007) as well as transcriptomic data (Bergholz et al., 2009) obtained from *P. articus* 273-4 revealed different adaptation mechanisms to permafrost environment, including modification of transport systems, changes in translation machinery, energy metabolism, and others.

The distribution and abundance of *Psychrobacter* exhibit geographic patterns. Biogeography on soils or sediments from polar, temperate and tropical environment have shown that *Psychrobacter* strains are more commonly found in the Polar Regions, but they can be detected in temperate and tropical sites. However, the occurrence of *Psychrobacter* populations outside cold environments were described as being associated to physicochemical conditions such as high salinity, presence of K^+ and alkaline pH, which are frequently found in Polar Regions as well (Rodrigues et al., 2008). *Psychrobacter* populations from Polar Regions are different from those found in temperate and tropical environments, which are similar to each other (Rodrigues et al., 2008).

1.2.2 Functional diversity

Most biochemical processes occur at surfaces or interfaces between different environments (Hardy, 1982). In the particular case of aquatic systems, the bacterial activity within the SML can mediate the air–sea exchange of reactive gases such as methane (CH_4), nitrous oxide (N_2O), carbon dioxide (CO_2) and carbon monoxide (CO) (Upstill-Goddard et al., 2003; Sabine et al., 2004; Conrad & Seiler, 1988).

However, although the common conclusion of several studies is that the bacterioneuston is involved in gas cycling, studies characterizing the diversity of bacterial genes that encode enzymes related to this function are still scarce. The diversity of functional genes that encode subunits of methane monoxygenase (*mmoX*) and carbon monoxide dehydrogenase (*coxL*) in estuarine SML compared to the corresponding UW samples were evaluated at the Blyth estuary (Cunliffe et al., 2008). The obtained results revealed that *mmoX* genes were less diverse in SML samples and were markedly different from *mmoX* genes in the UW. A very high diversity was found among the *coxL* genes in

both water layers. Nevertheless, a large number of unique *coxL* genes were detected in the SML and UW clone libraries. Therefore, these results indicate that the estuarine SML bacterial population may be specifically adapted for the consumption of these trace gases (Cunliffe et al., 2008).

The SML is subjected to intense solar radiation. Hence, studies regarding the resistance to ultra-violet radiation (UVR) on bacterioneuston communities are undoubtedly relevant. Even so, only a few studies have addressed this field so far and contradictory observations have been reported (Agogué et al., 2005b; Santos et al., 2011a; 2011b; 2012). Agogué and co-workers (2005b) observed no relationship between bacterial UVR resistance and water layers. Moreover, no significant differences were observed in the UVR inhibition on microbial activities of both communities (determined as [H^3] leucine incorporation). Among the highly resistant isolates, two dominant genera were identified: *Pseudoalteromonas* and *Alteromonas*. Although resistance patterns were similar in bacterioneuston and bacterioplankton isolates, the accumulation of exopolysaccharides and organic compounds in the SML may provide protection to bacterioneuston against high level of UVR (Elasri & Miller, 1999).

Effects of UVR on the abundance, diversity and activity of bacterioneuston and bacterioplankton communities from *Ria de Aveiro* (Portugal) were evaluated in a microcosm-based study (Santos et al., 2011a). After UVR exposure, bacterial abundance in both water layers decreased and DGGE profiles revealed greater reduction in the diversity of bacterioplankton compared to the bacterioneuston. On the other hand, heterotrophic activities were more affected in bacterioneuston indicating re-directioning of bacterioneuston metabolism towards stress defence/recovery strategies rather than the sustained heterotrophic metabolism.

Field studies conducted by the same authors (Santos et al., 2011b) revealed that bacterioneuston isolates were less sensitive and recovered more rapidly from UVR stress than bacterioplankton isolates. Additionally, for the first time, the response of individual bacterioneuston isolates to UVR exposure regarding the culturability, activity and metabolic recovery were evaluated.

Evidences were also gathered which indicated that bacterioplankton community was more affected in its bacterial abundance and DNA synthesis after UVR exposition, again indicating enhanced UVR tolerance of bacterioneuston (Santos et al., 2012). In terms of

structure, *Actinobacteria* increased in abundance in bacterioneuston, remaining un-affected in bacterioplankton (Santos et al., 2012).

There have been only few field studies concerning enzymatic activities in the SML (Kuznetsova & Lee, 2001; Mudryk & Skórczewski, 2000; 2004). As much of the dissolved and particulate organic carbons (DOM and POM) accumulate in the SML, studies into this field can shed light into organic matter degradation and nutrient cycling in aquatic ecosystems. Occurrence of lipolytic activity was measured in bacterioneuston and bacterioplankton isolates from the estuarine Lake Gardno (Mudryk & Skórczewski, 2000). Lipolytic bacteria were more numerous in bacterioneuston than in bacterioplankton in summer and autumn and reached a balance during spring season. Additionally, multiple-lipid decomposition was generally more common in bacterioneuston than bacterioplankton. Despite of that, levels of lipolytic activity were higher in bacterioplankton, suggesting that lipolytic activity in bacterioneuston community might be compromised by the stressful effect of solar radiation, accumulation of pollutants and fluctuations in salinity/temperature.

Differences between extracellular enzymatic peptide hydrolysis in the SML and the corresponding UW from Stony Brook Harbor (New-York) were investigated during one year (Kuznetsova & Lee, 2001). In overall, peptide hydrolysis was more effective in SML than UW. However differences between the two water layers were greater in winter time, probably reflecting seasonal variation of DOM enrichment in the SML.

Variations in hydrolytic activity of eight extracellular activities in SML and UW in three zones along the estuarine Lake Gardno were measured (Mudryk & Skórczewski, 2004). Significant differences in enzyme activity were observed, being more pronounced in the sea zone of the Lake Gardno, which is in fact more contaminated by sewage discharge from the holiday resort comparing to the less polluted mixed and freshwater zones. Additionally, the results indicated that the activity of esterase, aminopeptidase, α-glucosidase, β-glucosidase and β-lactosidase reached the highest values in surface layer, whereas lipase, phosphatase and chitinase showed maximum activity in UW (Mudryk & Skórczewski, 2004).

Bacteria that degrade PAHs in the estuarine SML from *Ria de Aveiro* (Portugal) were isolated and characterized. Among the PAH-degrading bacteria, *Pseudomonas* was dominant and screening for PAH dyoxigenases genes was only detected in two isolates

phylogenetically affiliated to *Pseudomonas* and an unknown species of the family Enterobacteriaceae. This result may indicate the presence of novel genes encoding PAH-degrading enzymes in this estuarine bacterioneuston (Coelho et al., 2010).

The common conclusion from these studies is that the bacterioneuston is closely involved in the cycling of at least some climatically active trace gases, and in degradation of organic matter and polutents promoting nutrient cycling in aquatic ecosystems.

Other functional aspects of bacterioneuston communities, which may be related to the specific physicochemical properties of this layer, have been evaluated. For example studies have been reported comparing the prevalence of bacterial antibiotic resistance (Hermansson et al., 1987; Mudryk & Skórczewski, 1998; 2009; Mudryk, 2002) or antibiotic production (Hakvåg et al., 2008) in the SML and UW. Antibiotic producers seem to be enriched in the SML: 80% of the *Streptomyces* isolated from the water interface from the Trondheim Fjord (Norway) exhibited antagonistic activity against non-filamentous fungus, Gram-negative, and Gram-positive bacteria (Hakvåg et al., 2008). Also, several studies have indicated that antibiotic resistant bacteria are more abundant in the SML. However this aspect has been poorly explored.

In this study an attempt to confirm the enrichment of antibiotic resistant bacteria in the SML of *Ria de Aveiro* was conducted (**Chapter 5**). In the same chapter the presence of antibiotic resistance genes and mobile gnetic elements was assessed. For this reason a general overview of antibiotic resistance in estuarine environments and specifically in the surface microlayer of estuaries is presented below.

1.2.2.1 Antibiotic resistance in estuarine environments

Given the fact that several anthropogenic activities are located nearby estuarine zones, the inappropriate disposal of their sub-products are frequently drained into the estuarine system, consequently this aquatic environment becomes a repository of persistent contaminants. The presence of toxic substances (although some of them quite diluted), changes in salinity and temperature due to the tides and the input of organic matter, are altogether factors of stress to which microbial cells have to respond very quickly. In that sense, an estuarine aquatic system exerts strong selective pressures, and in consequence,

the bacterial fraction (perhaps the most plastic part of the microbial communities) tends to evolve to higher levels of resistance.

In the past few decades the uncontrolled and extensive use of antibiotics in human and veterinary medicine, animal husbandry, agriculture and aquaculture has caused the increased introduction of those antimicrobial agents in the aquatic environment, including estuarine systems (Hirsch et al., 1998; Golet et al., 2002). Indeed, the presence of a variety of antibiotics in estuarine waters resulting from human activities from surrounding areas has already been reported (Xu et al., 2007; Su, 2008; Zou et al., 2011; Zheng et al., 2011; Hoa et al., 2011). Additionally, estuarine bacterial isolates have been analyzed for their antibiotic resistance and strains showing multi-resistance patterns were often observed (Henriques et al., 2006c; Laroche et al., 2009; Evangelista-Barreto et al., 2010; Zheng et al., 2011; Zhang et al., 2011).

Given the greater concentration of nutrients, pollutants (including the persistence of antimicrobial agents even at sub-inhibitory concentrations) and high microbial density in the estuarine systems and specifically in the SML, the genetic exchange may be facilitated and mobile genetic elements at high densities can accelerate gene recombination and transfer (Martinez, 2009).

For example, integrons are recognized as efficient structures for acquisition, expression and dissemination of antibiotic resistance genes (ARGs) (Rowe-Magnus & Mazel, 2002). Those structures are common in isolates from estuarine waters, carrying gene cassettes conferring resistance to a wide range of antibiotics (Rosser & Young, 1999; Henriques et al., 2006ab; 2008; Laroche et al., 2009). For example, class I integrons were detected in 3.6% of 3000 Gram-negative isolates from an estuary (Rosser & Young, 1999). Most of the variable regions in these integrons revealed that *aadA1* gene was predominant, although many other gene cassettes were also detected including those encoding resistance to beta-lactams (gene *oxa2*), erythromycin (gene *ereA*), chloramphenicol (genes *catB3* and *catB5*), aminoglycosides (genes *aadA2*, *aacA4* and *aacC1*) and trimethoprim (genes *dfr1a*, *dfrIIc*, *dfrV*, *dfrVII* and *dfrXII*). Moreover, a significant number of integrons were "empty". Thus, even in the absence of antibiotic selective pressures, empty integrons in bacteria from a natural habitat might persist (Rosser & Young, 1999). Similar results from highly polluted estuary in France were also reported (Laroche et al., 2009).

Other studies focused on the characterization of resistance to specific antibiotic classes in the estuarine environment. Beta-lactamases are enzymes able to hydrolyze the beta-lactam ring present in the structure of all beta-lactams (Bush, 1999). The hydrolysis of this ring renders the compounds inactive. The production of beta-lactamases constitutes the most effective and the most common mechanism of resistance to beta-lactams in Gram-negative bacteria (Kotra & Mobashery, 1998). Since beta-lactams are the most widely used antibiotics in human medicine and are also frequently used in veterinary and medicine, the dissemination of the production of beta-lactamases represents a great concern worldwide. DNA sequences putatively encoding enzymes included in the four classes of established families of beta-lactamases (Ambler, 1980) in the estuary of *Ria de Aveiro* (Portugal) were investigated (Henriques et al., 2006a). Obtained results reinforced the hypothesis that the environmental beta-lactamases gene pool comprises a complex mixture of ancient naturally occurring sequences and sequences that have been introduced or evolved more recently due to selective pressures resulting from human activities.

Later, Henriques and co-workers (2006b; 2008) have investigated the occurrence and diversity of integrons and resistance genes for resistance to beta-lactams and tetracycline in the same estuary. Comparing to their former investigation based on cultured-independent methods, they have concluded that the culture-dependent approach underestimated the prevalence of ARGs in environmental samples. On the other hand, the culture-dependent approach allowed to obtain complementary information concerning antibiotic resistance phenotypes and taxa of those estuarine isolates carrying ARGs and integrons. Those studies provided evidences that the estuarine environment plays a pivotal role on the maintenance and dissemination of ARGs.

More recently, ARGs have been recognized as new emerging contaminants in the environment (Pruden et al., 2006). Hence, based on the studies conducted so far it can be concluded that estuarine systems serve not only as important reservoirs for a variety ARGs (Henriques et al., 2006a), but also as dissemination vectors for spreading ARGs to open sea, thus promoting global pollution (Wells et al., 2007).

The occurrence of antibiotic resistant bacteria in estuarine environments along with the evidences that SML supports a biofilm rich in nutrients and densely colonized might indicate that SML constitutes a potential hot spot for horizontal gene transfer and also for the dissemination of antibiotic resistance justifying the need for further investigations.

Particularly studies focusing on the characterization of antibiotic resistance genes and resistant bacteria as well as on the mobile genetic elements in the bacterioneuston are needed. In the specific case of mobile genetic elements, new molecular variants of broad host range plasmids have already been isolated from SML samples retrieved from *Ria de Aveiro* estuary (Oliveira et al., 2012).

2. Scope of this thesis

2.1 Estuaries

Estuaries are semi-enclosed bodies of water where freshwater from rivers and a coastal stream merges with the ocean. In this mixing of waters with different salt concentrations, many chemicals substances accumulate in the water or sediments. Hence, physico-chemical and biological processes take place and consequently an unique habitat for birds, mammals, larvae (from fish and crustaceans), plants and wildlife species is formed (McLusky & Elliott, 2004).

Estuaries are also known for their filtering capacity determined by the existence of biologically active zones with high concentration of living organisms (Golubkov et al., 2001). However, due to the rapid population growth, uncontrolled urbanization of coastal areas worldwide and development of economically important harbours, estuaries have become affected by anthropogenic pressures resulting in ecosystem degradation, thus compromising their filtering and nursery capacities.

2.2 Sampling site: *Ria de Aveiro*

The estuary *Ria de Aveiro* has 47 km long, with a maximum width of 11 km, in the North-South direction, from the city of Ovar to Mira. In total this estuary has 11000 hectares, from which 6000 are always covered with water. In this system it debouches the Vouga, Antuã and Boco rivers, and it has only a single communication with the sea by a channel between Barra and S. Jacinto (Hall et al., 1985; Dias, 1999) (Figure 2.1). It is a mesotrophic estuarine system with a complex topography, being separated from the sea by a sandy boundary and presenting a complex net of internal canals. The water exchange with the ocean is 89 Mm^3 while the freshwater entrance media, during the equivalent wave period, is 1.8 Mm^3 (Almeida et al., 2001).

Over time, *Ria de Aveiro* has been the target of several pollutant discharges, being the main sources of contamination the sludge waste from Aveiro's city and the diffuse pollution associated to Aveiro's seaport activities, industrial explorations, aquaculture tanks and pollutants from farming fields nearby (Henriques et al., 2004).

Even so, this estuary has a great economical importance due to professional and recreational fishing and aquaculture explorations, which are being intensively developed in

the recent years. Recent efforts have been made to solve the pollution problem in order to recycle and preserve the water quality and the ecosystem health.

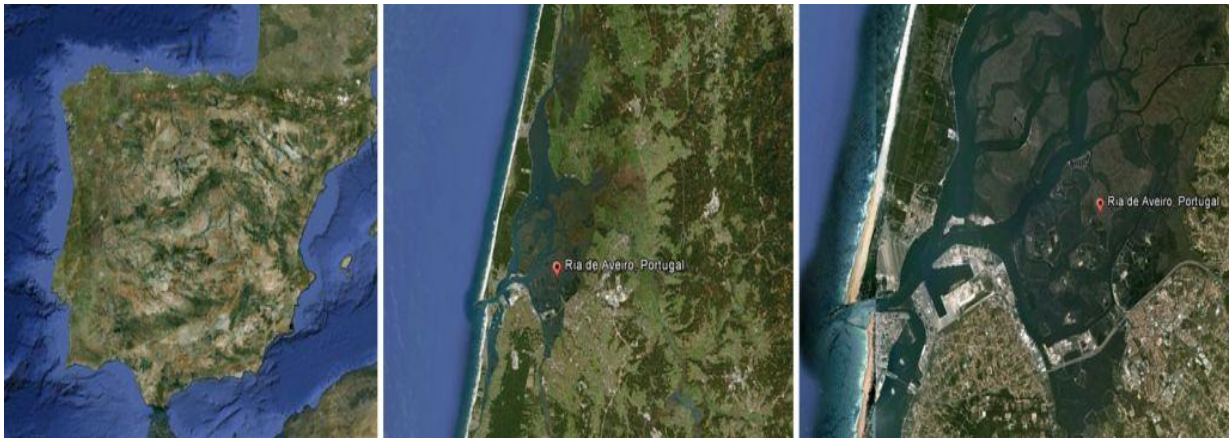

Figure 2.1 Estuary of *Ria de Aveiro*, Portugal

2.3 Objectives of this thesis

The main aims of this study were to characterise and compare the bacterioneuston and bacterioplankton communities inhabiting the estuarine environment *Ria de Aveiro*.

Specific aims were:

- In **Chapter 3** culture-dependent and culture-independent methodologies were applied to characterise the phylogenetic structure of the estuarine bacterioneuston and to compare it to the corresponding bacterioplankton.

- Also in **Chapter 3** the spatial and temporal short-term variability of both communities was assessed using the same methodologies.

- In **Chapter 4** the diversity of *Psychrobacter* populations inhabiting SML and UW in *Ria de Aveiro* were evaluated by analysing genus-specific clone libraries and fingerprinting-based methods. In this chapter we also analysed a collection of *Psychrobacter* strains obtained from both layers.

- In **Chapter 5** the prevalence of cultivable antibiotic-resistant bacteria in the SML and UW in *Ria de Aveiro* was assessed. Also the antibiotic resistance profiles of SML and UW isolates were compared.

- In **Chapter 5** we also investigated the occurrence of genes encoding antibiotic resistance as well as the presence and diversity of integrons in isolates from both water layers.

3. Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies

3.1 Introduction

A thin (roughly 1 to 1000 µm) surface film was reported to form at the interface between marine environments and the atmosphere (Liss et al., 1997). This physical boundary has commonly been designated as sea-surface microlayer (SML) albeit similar films occur at the surface of freshwater and estuarine systems (Cunliffe et al., 2008; Hervàs & Casamayor, 2009). SML ecological relevance is well recognized and derives from the fact that it covers about 70% of the Earth's surface and strongly impacts the exchange of gases and matter across the air-water interface (Maki, 2002; Zemmelink et al., 2005). Specifically, SML has a large effect on water–air exchange of gases directly related to climate changes, such as carbon monoxide, carbon dioxide, methane and dimethyl sulfide (Conrad & Seiler, 1988; Upstill-Goddard et al., 2003; Zemmelink et al., 2005; Cunliffe et al., 2008).

The SML is distinct from underlying waters (UW) in terms of its physical and chemical properties (Cunliffe et al., 2011; Liss et al., 1997). For example, this layer has been described as a place of accumulation of organic matter and of a variety of pollutants including hydrocarbons and heavy metals (Cincinelli et al., 2005; Cuong et al., 2008).

Based on these distinct characteristics it has been hypothesized that SML also constitutes a unique ecosystem, which includes distinct biological communities (Maki, 2002). The bacterial community present within this layer is usually referred to as bacterioneuston (Naumann, 1917). In the past, the SML has been reported to comprise more abundant and active bacterial communities than subjacent waters (Sieburth et al., 1976; Hardy, 1982). Regarding phylogenetic composition, while some authors reported considerable compositional differences between bacterioneuston and bacterioplankton (Franklin et al., 2005; Cunliffe et al., 2008) others did not find relevant dissimilarities (Agogué et al., 2005a; Obernosterer et al., 2008;). Inconsistencies between studies have been related to the use of different SML sampling devices (Cunliffe et al., 2009a; Cunliffe et al., 2011). Though, spatial and temporal factors might contribute to the unevenness in the SML formation and thickness and accordingly to the variability in the structure and functional properties of bacterioneuston (Peltzer et al., 1992; Santos et al., 2009).

Estuaries may represent one of the most favorable habitats for the establishment of distinct bacterioneuston communities since they consist on semi-enclosed water bodies, usually strongly influenced by anthropogenic activities, often becoming eutrophicated and concentrating high levels of pollutants (Keddy, 2000). In these systems, terrestrially derived material probably contributes significantly to the formation of distinct surface layers. In fact, within estuaries the occurrence of visible surface slicks is a common phenomenon (Liss et al., 1997).

The properties of SML may vary significantly along time and space. Therefore it can be hypothesized that the degree of similarity between adjacent bacterioneuston and bacterioplankton would differ between sampling sites and dates. To confirm this hypothesis, in this study, the phylogenetic composition of bacterioneuston and bacterioplankton communities from a highly polluted estuarine system (*Ria de Aveiro*, Portugal) was compared by combining culture-independent and culture-dependent approaches. Nearly all studies conducted in the last decade examined differences between SML and UW communities using culture-independent methods. However, specific properties of SML, namely accumulation of organic matter at this interface (Cunliffe & Murrell, 2009), may conduce to the establishment of a distinct and highly active community of culturable heterotrophic bacteria. Thus, this community was also considered during this study.

Other authors (Cunliffe & Murrell, 2009) have hypothesized that microorganisms with ability to form biofilms have a selective advantage in the gelatinous film that SML is. For that reason, efforts were made to verify this hypothesis in what concerns *Aeromonas* and *Pseudomonas*, two genera whose members are commonly found in estuarine waters and have been frequently associated with biofilm formation.

3.2 Material and methods

3.2.1 Site description and sample collection

Ria de Aveiro is a shallow estuary on the north-west coast of Portugal (40º38'N, 8"45'W), about 45 Km long and 8.5 Km wide (Figure 3.1). Samples were collected at three sites: *Cais do Chegado* (CC), where the main contamination sources are industrial effluents, *Costa Nova* (CN), mainly impacted by urban effluents, aquacultures and run-

off from agriculture fields and *Cais do Sporting* (CS) subjected to anthropogenic pressure mainly due to the presence of harbor facilities (Figure 3.1).

Samples were taken during 4 campaigns in May (C1), July (C2), September (C3) and October 2008 (C4), with gentle weather conditions and wind below 3-4 m/s. For each campaign, SML and UW samples were collected always at low tide, during day (maximum light) and during night (minimum light). A water layer of 60-100 µm was

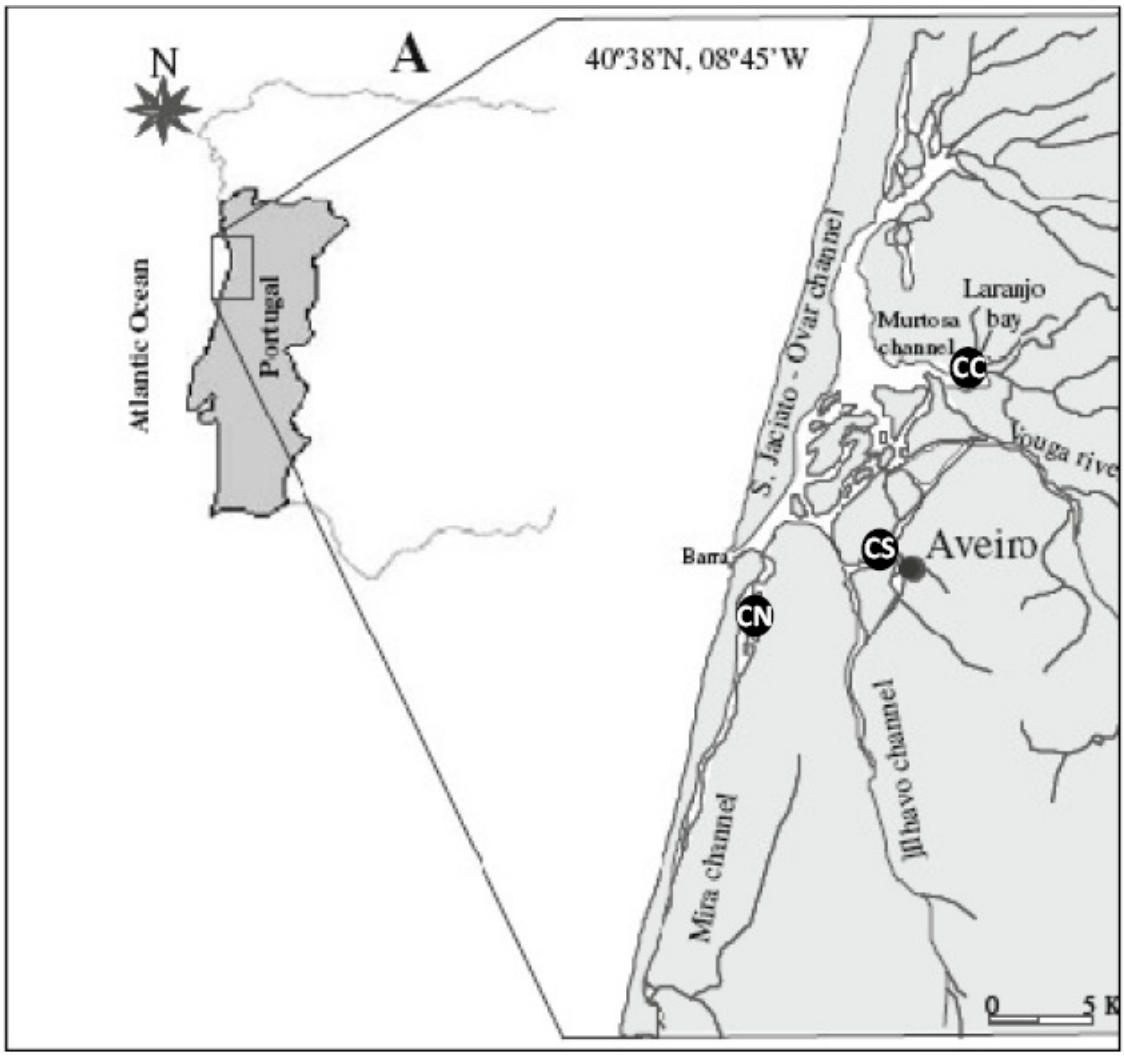

Figure 3.1 Map of *Ria de Aveiro* showing sampling sites in *Costa Nova* (CN), *Cais do Sporting* (CS) and *Cais do Chegado* (CC).

collected with a 0.25 m wide x 0.35 m long and 4 mm thick glass plate as previously described (Agogué et al., 2004; Santos et al., 2009). Prior to collection, the plate was washed with ethanol, sterile Milli-Q water and several times with the respective SML water. To minimize the contamination with UW, water draining from the plate for the first 20 s was rejected. Bacterioplankton was collected at approximately 0.4 m depth in 2 L autoclaved bottles. Samples were kept in cold and dark conditions during transport and were processed within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany) and measured using the Pratical Salinity Scale.

3.2.2 Enumeration, isolation and identification of culturable heterotrophic bacteria

Water samples were serially diluted in 0.9% NaCl and aliquots of 100 µL of each dilution were spread onto 4 replicate plates of Estuarine Agar (EA; Weiner et al., 1980) and GSP (Glutamate Starch Phenol Red Agar, *Pseudomonas*/*Aeromonas* selective agar) media. Colony-forming units (CFU) were counted after 3 (GSP plates) and 5 days (EA plates) of incubation at 22°C in the dark.

Colonies were randomly selected from each sample, purified and maintained on the same media. Approximately the same number of isolates was selected from each sample and, whenever possible, colonies were picked from plates corresponding to the same dilution. DNA extraction was performed using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania). Phylogenetic affiliation of the bacterial isolates was established by 16S rRNA gene sequence analysis. Amplification was performed with universal bacterial primers 27F and 1492R as described previously (Lane, 1991). PCR products were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) and used as template in the sequencing reaction carried out by the company Stab-Vida (Oeiras, Portugal). The sequence similarity search and phylogenetic affiliation were performed using the BLAST program (Altschul et al., 1997).

The 16S rRNA gene sequences from the SML and UW culture collections were processed by using the analysis pipeline on the Ribosomal Database Project (RDP) website (Cole et al., 2009; http://rdp.cme.msu.edu). Operational taxonomic units (OTUs) and rarefaction curves were calculated. Classical indices were used to estimate

richness (Chao's richness estimator) and diversity (Shannon-Wiener index) for each culture collection and to determine the similarity degree between culture collections (Bray-Curtis similarity index). All indices were calculated using the EstimateS software (version 7; available at http://viceroy.eeb.uconn.edu/estimates).

3.2.3 DNA extraction from water samples and community analysis

For DNA extraction 200 mL water samples from SML and UW were filtered through 0.2-µm-pore-size filters (Poretics Products). DNA extraction was performed using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania) as described previously (Henriques et al., 2004).

For each sample, DGGE was performed on DNA extracts to obtain bacterial and *Aeromonas*-specific molecular fingerprints of the SML and UW. The V3 region of bacterial 16S rRNA gene was amplified using the primers 338F and 518R as previously described (Henriques et al., 2006a). A fragment of the *gyr*B gene was amplified with *Aeromonas*-specific primers gyrB-F and gyrB-R following the protocol described by Calhau et al. (2010). For the *gyr*B gene, a reamplification approach was required to obtain sufficient PCR product for subsequent analysis. For this, a second PCR was conducted using 0.5µL of the first PCR product as template and the same primers and conditions.

A GC clamp was attached to the 5' end of the forward primers in order to prevent complete melting of the PCR products during subsequent DGGE analysis (Muyzer et al., 1993). The PCR reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using *Taq* polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

PCR products were directly applied onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) in 0.5X TAE buffer (20 mmol/L Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na_2EDTA) with urea and formamide as denaturants. Linear denaturing gradients ranged from 37 to 65% for 16S rDNA-DGGE and from 45 to 70% for gyrB-DGGE (100% corresponds to 7M Urea and 40% formamide). Electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad, USA) at 60°C; initially a constant voltage of 20 V was applied for 15 min followed by 75 V during 16 hours. After electrophoresis, the gels were stained for 5

min with ethidium bromide and then rinsed for 20 min in distilled water. Gel images were acquired using a Molecular Image FX apparatus (Bio-Rad Laboratories, Hercules, California, USA).

Gel images were analyzed with the Diversity Database^{TM} Software (Bio-Rad Laboratories, Hercules, California, USA). Band automatic assignment was carefully checked and, when necessary, manually edited. Taking into account the presence/absence and intensity of individual bands in each lane, a similarity matrix was constructed using the Bray-Curtis measure. Cluster analysis was performed using the PRIMER v6 software (Clarke & Gorley, 2001); the same software was used to calculate the Shannon–Wiener index (H'). Dendrograms were generated using the group average method.

3.2.4 Identification of DGGE bands

SML-specific bands, UW-specific bands and bands displaying different intensities in SML and UW profiles were selected for determination of its nucleotide sequence. Bands were aseptically excised, re-amplified and checked for accuracy in DGGE gels. The corresponding PCR products were cloned using a TA cloning kit (Invitrogen, California, USA) according to the manufacturer's instructions. Subsequently, at least six inserts were checked by PCR-DGGE and subjected to sequencing analysis as described above, using vector-specific primers. Band sequences were compared to the GenBank nucleotide data library using the BLAST software (Altschul et al., 1997) in order to determine their closest phylogenetic relatives.

3.2.5 Nucleotide sequence accession numbers

Sequences representing culturable OTUs were deposited in GenBank under the following accession numbers: JQ072029 to JQ072088. Sequences from DGGE bands were deposited under the following accession numbers: JQ237824 to JQ237846.

3.3 Results

3.3.1 Abundance and phylogenetic diversity of heterotrophic bacteria

The average number of CFUs per mL was highly variable between sites and campaigns (Figure 3.2). However, the mean CFU concentrations obtained for the SML samples were usually 2 to 8 times (and exceptionally up to 75 times) higher than the mean concentration of CFU in the UW samples. These differences were consistently more pronounced in site CN and in the third campaign.

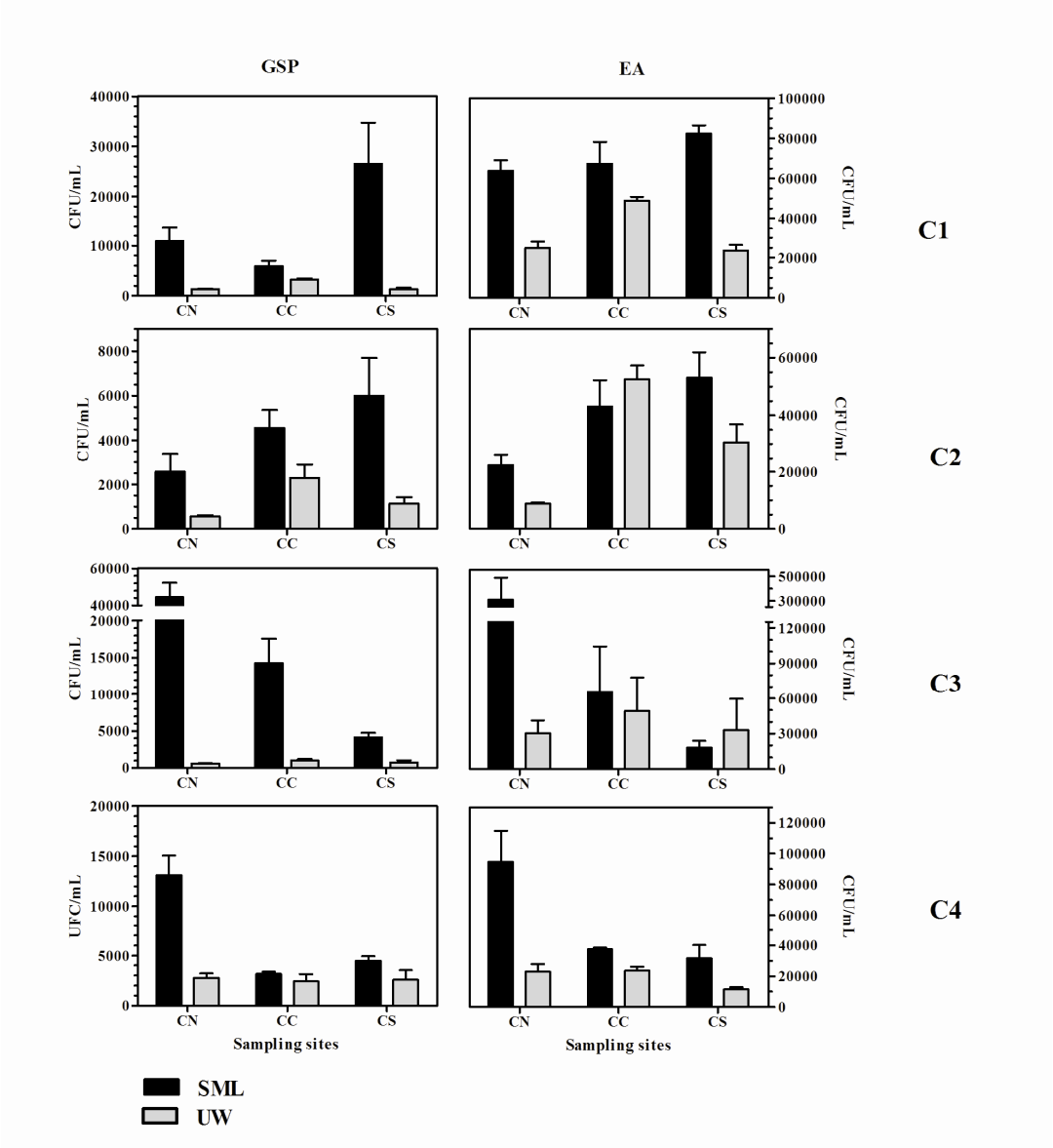

Figure 3.2 Fluctuations in mean CFU concentrations (CFU/mL) for SML and UW samples collected in 4 campaigns (C1 to C4) and plated in *Pseudomonas/Aeromonas* selective agar (GSP) and estuarine agar (EA) media.

A total of 352 isolates were recovered from water samples, being 168 from SML samples and 184 from UW samples. These isolates, based on identity criteria of >97% at the 16S rRNA gene sequence, could be categorized into 60 operational taxonomic units (OTUs) (Table 3.1). Two OTUs were dominant and included 39% of the isolates from both the SML and UW samples. OTUs containing a single isolate represented 46.7% of the total OTUs. A similarity value of 69% between the two culture collections was obtained using the Bray-Curtis index.

Based on phylogenetic analysis bacterial isolates were assigned to five phyla within the domain Bacteria, namely *Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, and *Actinobacteria*, and represented 20 orders (Figure 3.3; Figure 3.4). The positively identified strains belonged to 43 bacterial genera (Table 3.1). Additionally, isolates included into 3 OTUs (isolated from both layers) could not be identified at genus level and were affiliated with families *Enterobacteriaceae* (2 OTUs) and *Micrococcaceae* (1 OTU). The most common genera were *Psychrobacter* (89 isolates) and *Acinetobacter* (58 isolates). Bacteria belonging to the genera *Pseudomonas* (43 isolates), *Aeromonas* (25 isolates) and *Shewanella* (22 isolates) were also frequently isolated. Most pronounced differences between SML and UW samples were observed for order *Actinomycetales* (Figure 3.3; Table 3.1) namely for genera *Agrococcus* (12 isolates from UW) and *Kocuria* (5 isolates from UW), and for genus *Vibrio* (8 isolates from UW and 1 isolate from SML). On the other hand one of the OTUs assigned to the *Pseudomonas* genus was more abundant in the SML (20 SML isolates and 7 UW isolates) (Table 3.1).

3.3.2 DGGE analysis of bacterial community structure

DGGE fingerprinting was used to compare the bacterial community structure in SML and UW samples. During preliminary experiments, DNA extractions and subsequent PCR and DGGE analysis were carried out in triplicate. No detectable differences between profiles were observed. Also, analysis of DGGE profiles revealed negligible variability (<6%) between samples (n=5) collected within a limited area of approximately 10 m^2 for each sampling site (data not shown).

Subsequently, the sampling sites were visited four times during the warm season (from May to October) and temperature and salinity values for each sampling site/date

are presented in Table 3.2. As previously described for this estuarine system (Henriques et al., 2006a), temperature values were relatively stable in time and space while clear differences in salinity values between sampling sites and sampling dates were frequently observed (Table 3.2).

Table 3.1.Summary of bacteria isolated from SML and UW samples.

Phylogenetic group	Representative isolate[a, b] (accession nº)	Closest Relatives (accession nº)	Sequence identity (%)	Number of isolates in OTU[c]	
				SML	UW
Agrococcus	ENNP5_III (JQ072029)	*Agrococcus citreus* (AB279547)	99	0	12
Arthrobacter	ENDN1_III (JQ072030)	*Arthrobacter agilis* (EU730943.1)	98	1	0
	ENNN6_I (JQ072031)	*Arthrobacter arilaitensis* (EU240951.1)	100	1	1
Brevibacterium	ECNP2_I (JQ072032)	*Brevibacterium* sp.(FN392692.1)	99	0	1
Corynebacterium	ECNN8_I (JQ072033)	*Corynebacterium casei* (DQ361013.1)	99	1	0
Kocuria	ESDP1_III (JQ072034)	*Kocuria* sp. (FJ889675.1)	100	0	5
Microbacterium	ENNP2_I (JQ072035)	*Microbacterium* sp. (FJ765512.1)	99	0	1
Micrococcus	ENDN2_III (JQ072036)	*Micrococcus* sp. (FJ607363.1)	99	2	2
Micrococcaceae	ESNP6_II (JQ072037)	*Micrococcus* sp. (FJ607363)	99	2	6
Aerococcus	GCNN9_I (JQ072038)	Uncultured bacterium (GQ091598.1)	100	1	0
Bacillus	ESDN8_II (JQ072039)	*Bacillus cereus* (EF488087.1)	99	2	0
	ENDP2_I (JQ072040)	*Bacillus pumilus* (GU125637.1)	100	0	1
	ESDN10_III (JQ072041)	*Bacillus* sp. (AF440439.2)	99	1	0
Exiguobacterium	ESDP2_III (JQ072042)	*Exiguobacterium homiense* (FJ999945.1)	100	0	2
Planococcus	ENDN3_II (JQ072043)	*Planococcus* sp. (FJ237405.1)	99	2	0
Staphylococcus	GSDN10_II (JQ072044)	*Staphylococcus equorum* (EU855190.1)	100	1	2
Cyclobacterium	ENNN10_III (JQ072045)	*Cyclobacterium amurskyense* (FJ229465.1)	100	1	0
Algoriphagus	ECNP10_I (JQ072047)	*Algoriphagus aquatilis* (EU313811.1)	97	0	1
Leeuwenhoekiella	ENDP4_III (JQ072048)	Uncultured *Leeuwenhoekiella* (FN433319.1)	98	0	1
Flavobacterium	GNNN5_III (JQ072049)	*Flavobacterium* sp. (AM934639.1)	97	1	0
Olleya	ESDN4_II (JQ072050)	*Olleya marilimosa* (FJ015035.1)	100	1	0
Deinococcus	ESNP7_II (JQ072051)	*Deinococcus radiopugnans* (NR_026403.1)	99	0	1
Brevundimonas	GSDP8_I (JQ072052)	*Brevundimonas* sp. (FJ544245.1)	100	0	1
	GCNP1_II (JQ072053)	*Brevundimonas* sp. (DQ177489.1)	100	0	2
	GNDN8_III (JQ072054)	*Brevundimonas* sp. (DQ310472.1)	99	1	0
Erythrobacter	ENDN8_III (JQ072055)	*Erythrobacter citreus* (EU440970.1)	100	3	1
Agrobacterium	GCNN2_III (JQ072056)	*Agrobacterium tumefaciens* (FJ785222.1)	99	1	0
Pseudorhodobacter	ENDN8_I (JQ072057)	*Pseudorhodobacter incheonensis* (DQ001322.1)	100	2	2
Paracoccus	ESDP1_II (JQ072058)	*Paracoccus* sp. (AY167832.1)	99	0	2
Devosia	ECDN8_I (JQ072046)	*Devosia* sp.(FR731130.1)	97	1	0
Alcaligenes	GSNN1_I (JQ072059)	Uncultured *Alcaligenes* sp. (DQ168833.1)	99	2	1

Achromobacter	GNDN8_I (JQ072060)	*Achromobacter* sp. (GU138383.1)	97	1	0
Comamonas	GCNN4_I (JQ072061)	Uncultured bacterium (EU468035.1)	99	1	0
Delftia	GCNP4_II (JQ072062)	*Delftia* sp. (AB461757.1)	100	0	1
Arcobacter	GCDN6_III (JQ072063)	*Arcobacter* sp. (EF419216.1)	98	1	0
Aeromonas	GNDP3_I (JQ072064)	*Aeromonas hydrophila* (GQ470995.1)	100	14	11
Alteromonas	ESDN8_III (JQ072065)	*Alteromonas* sp. (EF061431.1)	99	1	0
Shewanella	GCDN4_III (JQ072066)	*Shewanella* sp. (FJ025779.1)	99	1	1
	ESDN7_III (JQ072067)	*Shewanella* sp. (EU979479)	98	11	9
Marinobacter	ECDP4_III (JQ072068)	*Marinobacter* sp. (FJ903190.1)	99	0	1
Pseudoalteromonas	ECNP10_III (JQ072069)	Uncultured *Pseudoalteromonas* (FJ695595)	100	1	1
	ESNP3_I (JQ072070)	*Pseudoalteromonas* sp. (AM913917.1)	99	0	1
Rheinheimera	ENNN3_I (JQ072071)	*Rheinheimera* sp. (AM110966.1)	99	2	4
Erwinia	ECDN2_I (JQ072072)	*Erwinia* sp.(AY690711.1)	99	3	0
Halomonas	GSNN9_III (JQ072073)	*Halomonas* sp. (FJ386522.1)	100	2	0
Marinomonas	GNNN6_I (JQ072074)	*Marinomonas* sp. (AY745826.1)	99	1	2
Acinetobacter	ENDN8_II (JQ072075)	Uncultured *Acinetobacter* (DQ234186.2)	99	23	25
	ECDP6_I (JQ072076)	*Acinetobacter* sp. (AF336348.1)	99	0	2
Pseudomonas	GNDP9_III (JQ072077)	*Pseudomonas cf. stutzeri* (AJ244724.1)	99	6	10
	GCDN9_III (JQ072078)	*Pseudomonas* sp. (GQ868355)	100	20	7
	GCNP2_III (JQ072079)	*Pseudomonas* sp. (AB461633.1)	100	0	1
Psychrobacter	GNDP2_II (JQ072080)	*Psychrobacter faecalis* (EU370413.1)	100	43	46
Vibrio	GNNN3_III (JQ072081)	*Vibrio* sp. (AM913925.1)	100	1	5
	GNNP7_III (JQ072082)	*Vibrio diazotrophicus* (NR_026123.1)	100	0	2
	ECNP1_III (JQ072083)	*Vibrio* sp. (AM902263)	99	0	1
Lysobacter	GCNP3_II (JQ072084)	*Uncultured bacterium* (JF168457.1)	99	0	1
Stenotrophomonas	GCDP10_III (JQ072085)	*Stenotrophomonas rhizophila* (GQ359325.1)	100	3	1
	GCDP4_I (JQ072086)	*Stenotrophomonas* sp. (EU073094.1)	100	0	1
Enterobacteriaceae	ENDP9_III (JQ072087)	*Hafnia alvei* (DQ412565.1)	99	3	2
	GNDN3_I (JQ072088)	Uncultured bacterium (GQ069695.1)	99	3	4

[a] Isolates were assigned a code where first letter represents the culture medium (**G**SP or **E**A), the second letter represents the sampling site (C**C**, C**N** or C**S**), the third letter represents the sampling period (**N**ight or **D**ay) and the fourth letter represents the sampled community (**N**euston or **P**lankton). Letters are followed by an arbitrary number (1 to 10) and the field survey number (I, II or III).

[b] One isolate was chosen to represent each defined OTU and the corresponding 16SrDNA sequence was deposited in the GenBank database.

[c] The data represent the number of isolates that were assigned to a particular OTU within SML and UW samples.

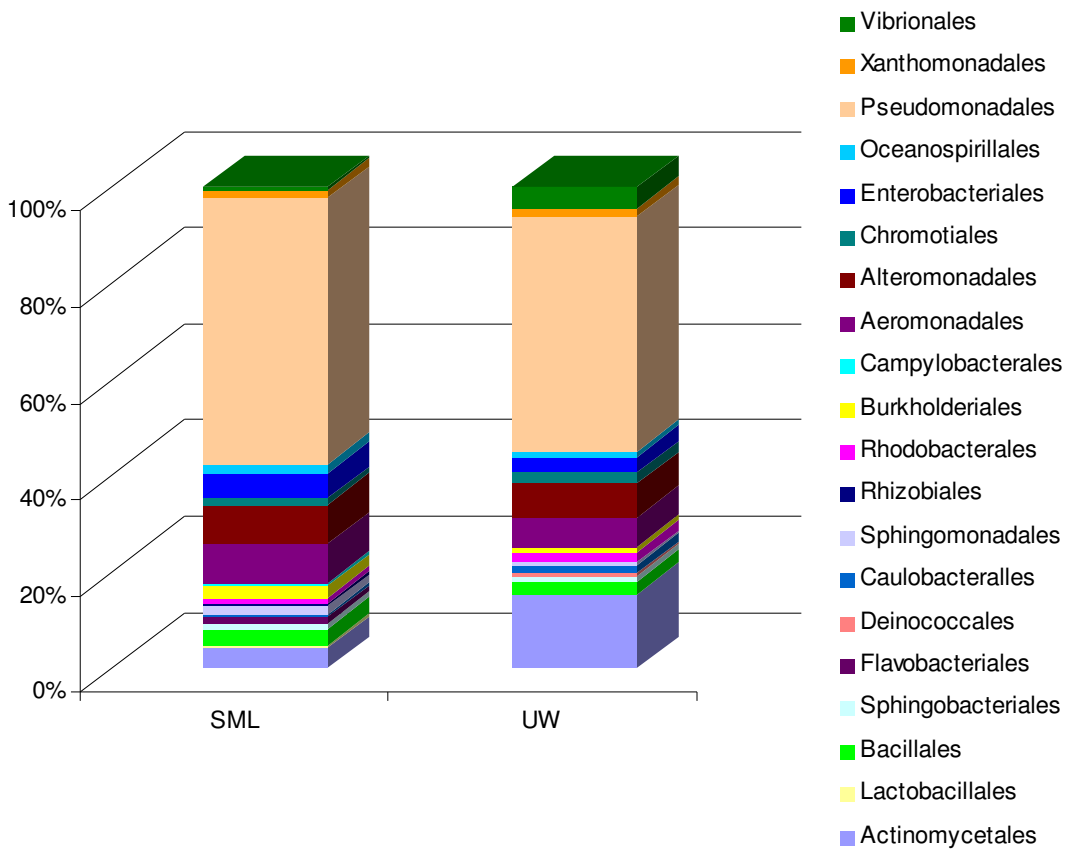

Figure 3.3 Stacked columns comparing the contribution at order level to the composition of culturecollections representing UW and SML samples.

Table 3.2. Values of temperature (ºC) and salinity measured during all campaigns in all sampling sites at day and night period.

Sampling site	Campaign	Temperature (ºC)		Salinity	
		Day	Night	Day	Night
CN	C1	20.2	20.6	20.6	20.8
	C2	20.2	20.6	23.5	23.5
	C3	19.6	20.5	27.7	27.5
	C4	19.4	19.8	27.9	27.8
CC	C1	21.3	21.7	16.6	16.6
	C2	21.3	21.7	18.7	18.7
	C3	20.9	19.8	28.9	29.5
	C4	21.6	19.6	29.7	30.1
CS	C1	19.7	20.8	26.1	26.1
	C2	19.7	20.8	29.2	29.3
	C3	18.8	18.8	33.1	33.3
	C4	19.0	19.2	33.1	33.1

16S rDNA DGGE profiles are shown in Figure 3.5. The number of DGGE bands detected in each profile ranged from 36 to 46 in SML samples and from 35 to 46 in UW samples. Also, overall community diversity did not consistently differ between SML and UW samples: the range of the Shannon–Wiener index values was from 1.52 to 1.61 (mean 1.55) for SML communities and from 1.48 to 1.60 (mean 1.55) for UW communities. Finally, for each campaign, a high number of common bands between all profiles could be identified (Figure 3.5).

In fact, cluster analysis showed that the similarity between all profiles was generally high (>50% according to Bray-Curtis measure; Figure 3.5). However, in spite of a number of exceptions, for each campaign, samples from the same sampling site clustered primarily by layer (samples collected from each layer during day and night frequently clustered together). Well-defined spatial-driven clusters, which included all samples collected from each site, were observed for the second campaign (sites CC and CN) and for the third campaign (sites CC and CS). On the other hand, temporal compositional shifts were clearly identified for all sampling sites.

Fourteen bands were excised from the 16S rDNA DGGE profiles obtained from SML samples (Figure 3.5). Bands 1, 2, 4, 5, 6, 8 and 12 were also detected in UW profiles but were more pronounced in SML profiles while bands 3, 7, 9, 10, 13 and 14 were only detected in SML samples. Taking into account band intensity, some of the excised bands seem to represent dominant phylotypes (1, 4, 5, 6, 7, 8 and 10). Eight out of 14 bands were excised from profiles from site CS, including 4 SML-exclusive bands (3, 7, 13 and 14). The recovered sequences had high similarity to known bacteria or environmental sequences. However, it was interesting that 4 out of 6 SML-exclusive bands shared only 94% identity with previously reported sequences. Most of the sequenced bands had closest relatives originating from aquatic environments (Table 3.3). Phylogenetically, the sequences were distributed in four groups: *Cyanobacteria* (3 bands), *Bacteroidetes* (2 bands), *Actinobacteria* (1 band) and *Gammaproteobacteria* (3 bands). Four clones (2, 3, 4 and 7) could only be assigned to domain Bacteria and 1 sequence was most closely related to chloroplast sequences.

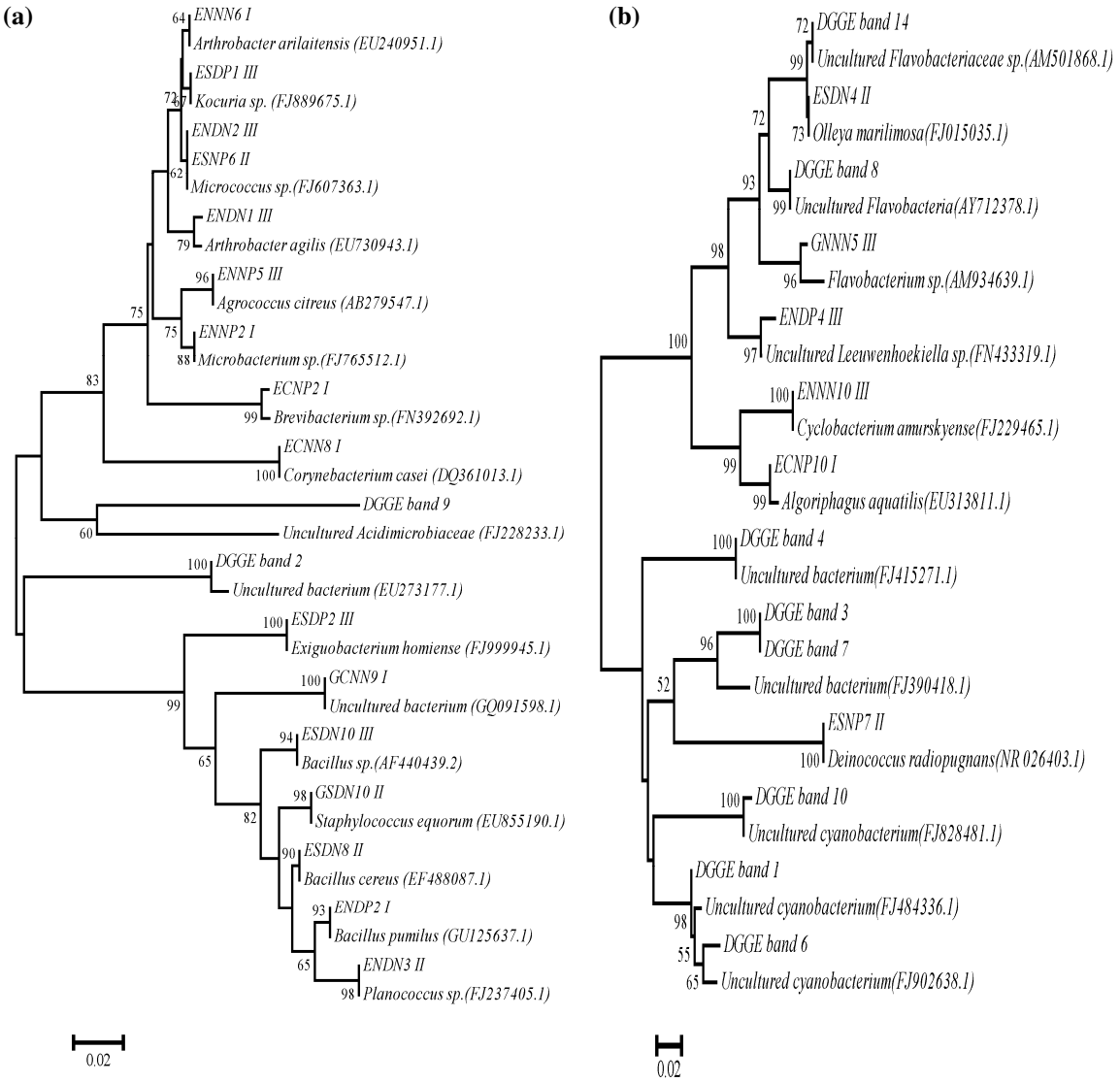

Figure 3.4 Neighbour joining phylogenetic trees showing the relationships among 16S rRNA gene sequences from bacteria isolated during this study, DGGE bands and sequences obtained from GenBank (accession numbers of these sequences are given in parentheses) affiliated with Gram-positive bacteria (a), other phylogenetic groups (b) and Proteobacteria (c). Distances were corrected using the Jukes-Cantor method. Bootstrap values > 50% are indicated at the nodes for 1000 replicates. The bar indicates the estimated sequence divergence.

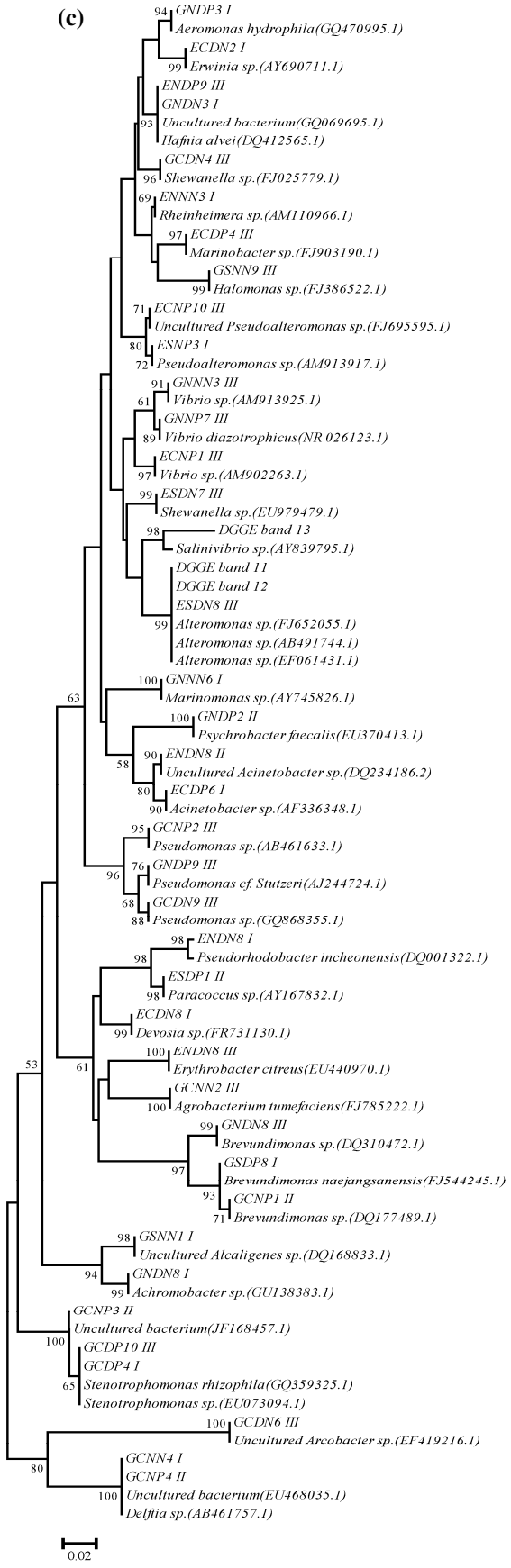

Figure 3.4 Continued

3.3.3 DGGE analysis of aeromonads community structure

Aeromonas-specific DGGE profiles were obtained for samples collected during the first, second and fourth campaign (Figure 3.6). The number of DGGE bands detected in each profile ranged from 19 to 25 in SML samples and from 15 to 24 in UW samples. According to cluster analysis, the variability between these profiles was higher (>70%) than between bacterial profiles (>50%) (Figure 3.6). However, no systematic difference was observed between SML and UW *Aeromonas* communities. Also, no strong spatial-driven differences were observed except for the first campaign where samples from CC and CN formed well-defined clusters.

From *Aeromonas*-specific profiles nine bands were sequenced. Six bands were retrieved from UW and 3 from SML profiles (Figure 3.6). Most (5 out of 9) bands were selected from sampling site CS. Two bands (Aer–3, Aer–4) appear to be exclusive from SML samples and 5 bands were putative UW-exclusive (Aer–5, Aer–6, Aer–7, Aer–8 and Aer–9). All band sequences affiliated with members of the *Aeromonas* genus and all sequences were at least 94% identical to the database entries (Table 3.3).

3.4 Discussion

3.4.1 General features of *Ria de Aveiro* bacterial communities

The analysis of DGGE profiles obtained during this study in terms of number of bands and band positions revealed the presence of complex and highly stable bacterioplankton and bacterioneuston in *Ria de Aveiro*. The observed spatial stability is in accordance with previous studies, which reported the dominance of several widespread phylotypes in this estuary (Henriques et al., 2006a). However this apparent stability may be restricted to dominant groups since 16S rDNA DGGE is able to retrieve only sequences that are present in at least 0.5-1% of the total cells in the sample (Muyzer et al., 1993).

The culture-based approach led to the successful cultivation of a considerable diversity of heterotrophic bacteria included in 5 phyla. The analysis of the 16S rRNA gene sequences indicates that potentially new putative species have been cultured since a number of sequences shared <98% identity with any previously cultured isolate.

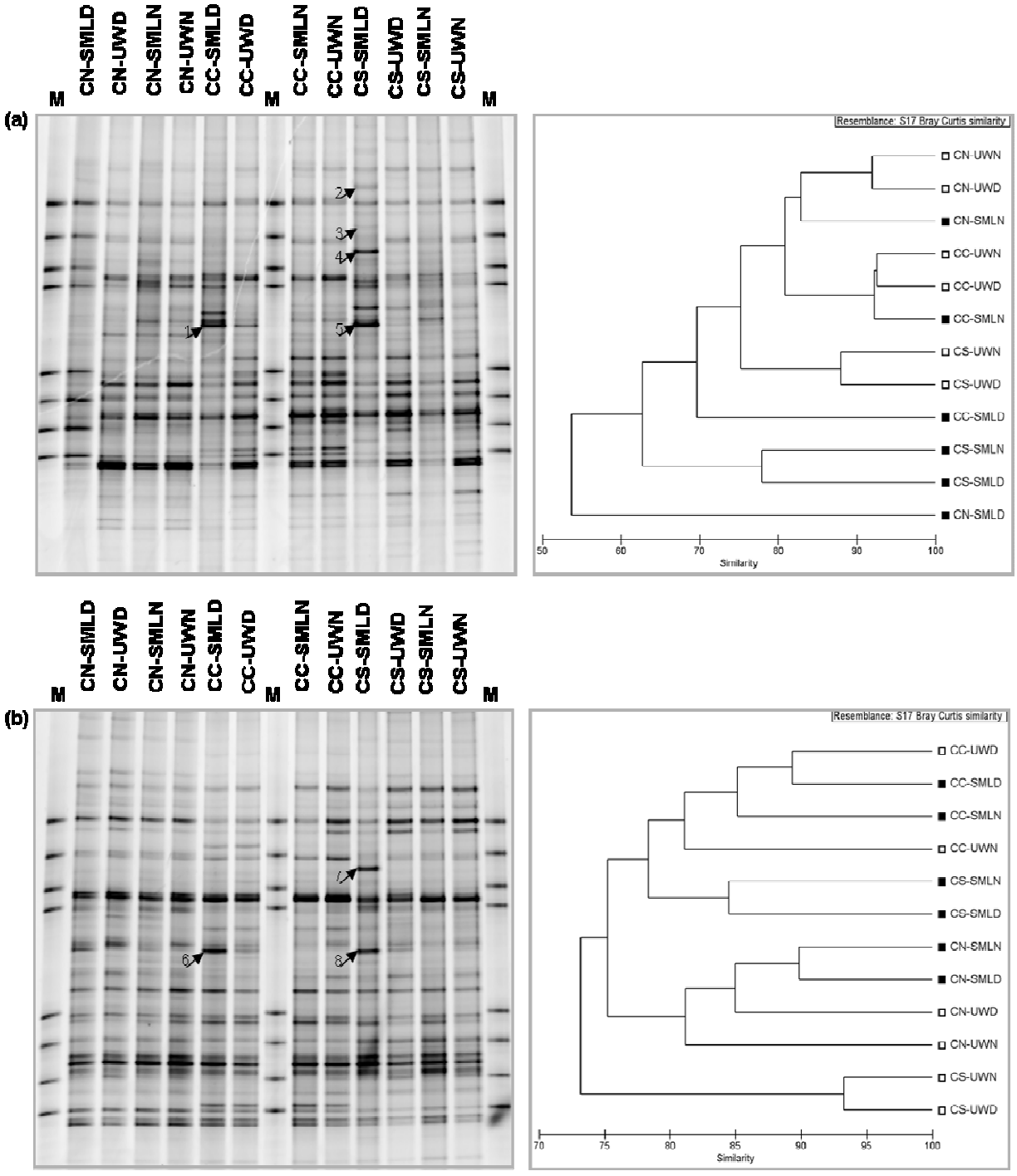

Figure 3.5 DGGE analysis of the bacterial diversity in UW and SML samples collected in May (a), July (b), September (c) and October (d). Lane labels indicate samples collected from sampling sites CN, CC and CS, during day (D) and night (N). Arrows indicate DGGE bands for which the DNA sequence was determined. For each gel a 16S rDNA-based cluster diagram is presented

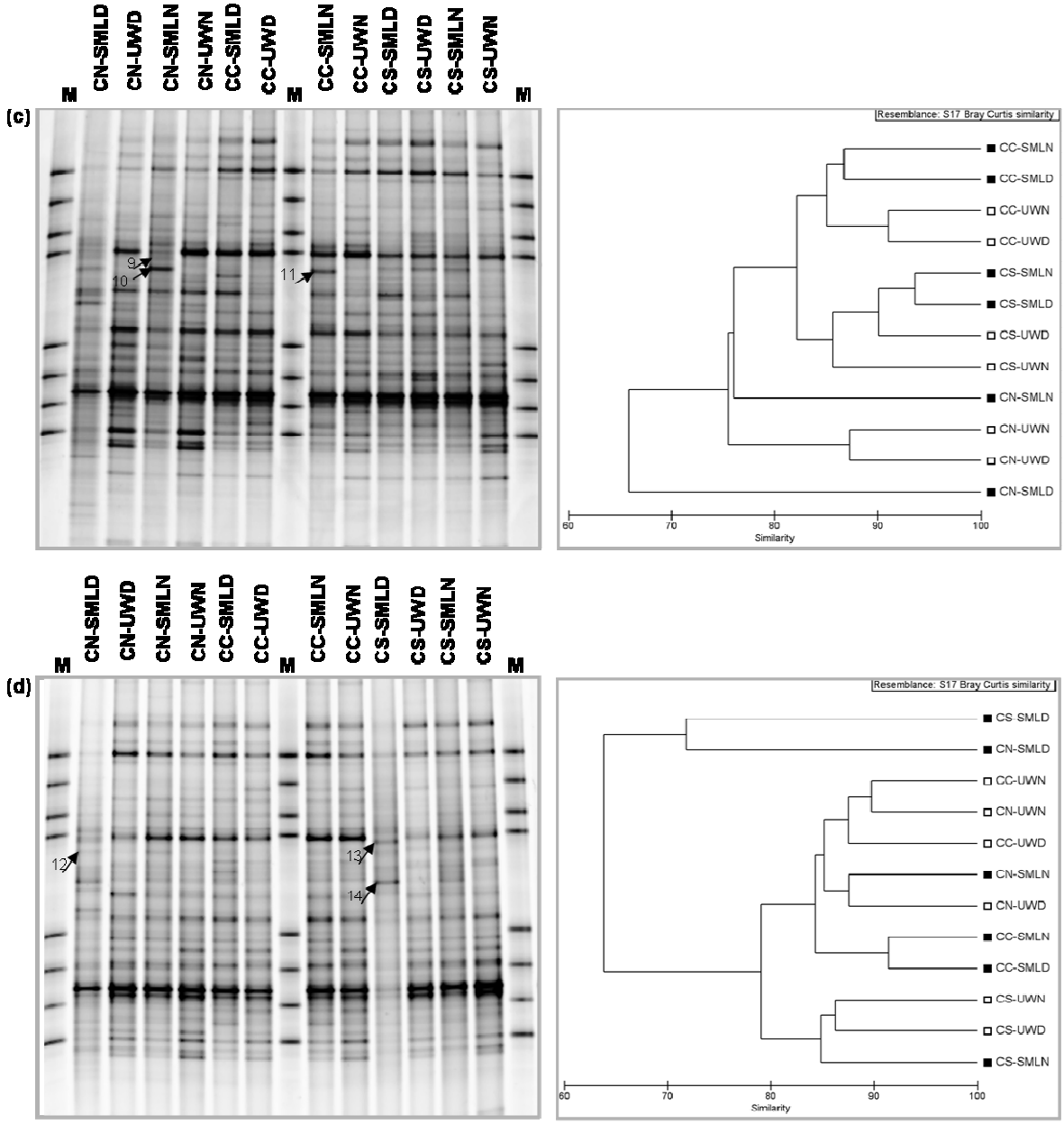

Figure 3.5 Continued

Additionally several sequences affiliated with previously observed but not yet formally described taxa. In general, the dominant bacterial groups found in SML and UW culture collections were similar to those commonly cultivated from estuarine water (Frette et al., 2004; Agogué et al., 2005a).

The most frequently retrieved genera from both the SML and UW were *Psychrobacter* and *Acinetobacter* followed by *Pseudomonas*, *Aeromonas* and *Shewanella*. Most of the aeromonads were isolated from the selective medium but representatives of the other genera were abundant in estuarine agar indicating that they most likely represent relevant members of the culturable community.

The abundance of *Psychrobacter* was unexpected in view of the present knowledge about this genus: these microorganisms have been isolated mainly from cold environments and were thought to be only marginally successful in other environments (Rodrigues et al., 2009). Contamination with hydrocarbons has been related to *Psychrobacter* enrichment in several previously published studies (Harwati et al., 2007; Prabagaran et al., 2007; Giudice et al., 2010).

In our study, *Psychrobacter* isolates were predominantly retrieved from the site CS, mainly impacted by harbor activities and where contamination with hydrocarbons has been previously confirmed (Coelho et al., 2010).

Table 3.3 Phylogenetic affiliation of 16S rDNA (1 to 14) and GyrB (Aer-1 to Aer-9) sequences retrieved from DGGE bands.

Band n°	Accession n°	Sample	Closest relative (accession n°)	Origin	Phylogenetic affiliation	Similarity (%)
1	JQ237824	CC-SMLD	Uncultured cyanobacterium (FJ484336.1)	Phreatic sinkhole - Mexico	Cyanobacteria	100
2	JQ237825	CS-SMLD	Uncultured bacterium (EU273177.1)	Taihu lake - China	Bacteria	99
3	JQ237826	CS-SMLD	Uncultured bacterium (FJ390418.1)	Miyun water reservoir - China	Bacteria	94
4	JQ237827	CS-SMLD	Uncultured bacterium (FJ415271.1)	Guanting water reservoir - China	Bacteria	100
5	JQ237828	CS-SMLD	Chloroplast (AY663923.1)	Coastal water - China	Chloroplast	99
6	JQ237829	CC-SMLD	Uncultured cyanobacterium (FJ902638.1)	Phreatic Sinkholes - Mexico	Cyanobacteria	98
7	JQ237830	CS-SMLD	Uncultured bacterium (FJ390418.1)	Miyun water reservoir - China	Bacteria	94
8	JQ237831	CS-SMLD	Uncultured Flavobacteria (AY712378.1)	Salt marsh - Sapelo Island - USA	Bacteroidetes	100
9	JQ237832	CN-SMLN	Uncultured Acidimicrobiaceae (FJ228233.1)	Sediments of acidic mine pit lake Brandenburg - Germany	Actinobacteria	94
10	JQ237833	CN-SMLN	Uncultured cyanobacterium (FJ828481.1)	Eutrophic lake -USA	Cyanobacteria	99
11	JQ237834	CC-SMLN	*Alteromonas* sp (FJ652055.1)	Seawater desalination membrane	γ-Proteobacteria	100
12	JQ237835	CN-SMLD	*Alteromonas* sp. (AB491744.1)	Settlement substrata - Japan	γ-Proteobacteria	100
13	JQ237836	CS-SMLD	*Salinivibrio* sp. (AY839795.1)	Solar salterns - Korea	γ-Proteobacteria	94
14	JQ237837	CS-SMLD	Uncultured Flavobacteriaceae (AM501868.1)	Lagoon anoxic sediments - Italy	Bacteroidetes	100
Aer-1	JQ237838	CN-UWN	*Aeromonas veronii* (AB473092.1)	Sputum	γ-Proteobacteria	98
Aer-2	JQ237839	CS-SMLD	*Aeromonas hydrophila* (AY968042.1)	Activated sludge - China	γ-Proteobacteria	99
Aer-3	JQ237840	CN-SMLN	*Aeromonas allosaccharophila* (FJ238496.1)	Wastewater treatment plant - Portugal	γ-Proteobacteria	96
Aer-4	JQ237841	CC-SMLD	*Aeromonas* sp. ER.1.21 (FJ238503.1)	Wastewater treatment plant - Portugal	γ-Proteobacteria	99
Aer-5	JQ237842	CS-UWD	*Aeromonas veronii* (AB473092.1)	Sputum	γ-Proteobacteria	98
Aer-6	JQ237843	CS-UWN	*Aeromonas bestiarum* (AY987521.1)	Ditch water	γ-Proteobacteria	95
Aer-7	JQ237844	CS-UWN	*Aeromonas eucrenophila* (AY101776.1)	Fresh water fish	γ-Proteobacteria	94
Aer-8	JQ237845	CC-UWD	*Aeromonas caviae* (JF938610.1)	Wastewater treatment plant - Portugal	γ-Proteobacteria	98
Aer-9	JQ237846	CS-UWN	*Aeromonas veronii* (AB473092.1)	Blood	γ-Proteobacteria	98

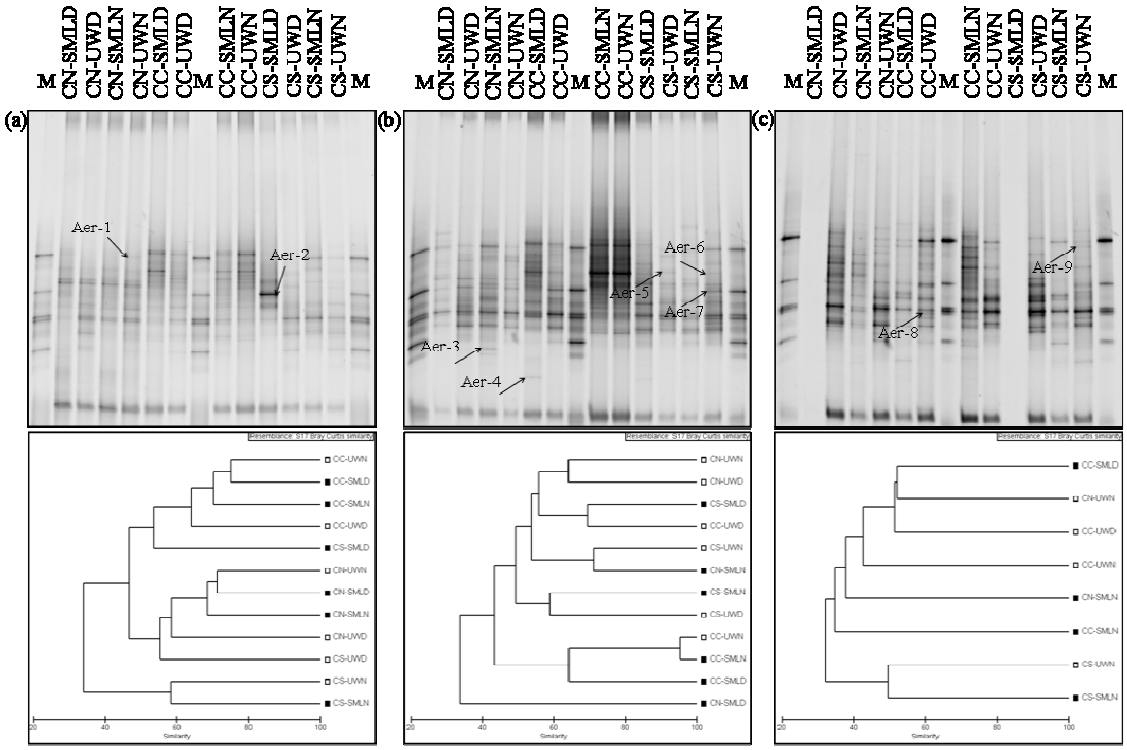

Figure 3.6 DGGE analysis of *Aeromonas*-specific *gyrB* amplicons in UW and SML samples collected in May (a), July (b) and October (c). Lane labels indicate samples collected from sampling sites CN, CC and CS, during day (D) and night (N). Arrows indicate DGGE bands for which the DNA sequence was determined. For each gel a 16S rDNA-based cluster diagram is presented.

The ability of members of the genus *Acinetobacter* to survive under dry conditions and to be easily transported by air is well known (Hervàs et al., 2009; Reche et al., 2009). Additionally, the presence of airborne *Acinetobacter*-related bacteria in SML has previously been suggested (Hervàs & Casamayor, 2009). However, despite being a dominant genus in our samples, differences between SML and UW in terms of abundance or diversity of *Acinetobacter* were not confirmed in this study.

Debates about the shortcomings associated with culture-dependent and culture-independent studies have been frequently carried out and reported. Because most of all the studies that examined differences between SML and UW communities in the last decade used culture-independent methods we decided to combine both approaches.

Despite the shortcomings commonly associated with 16S rDNA DGGE (Henriques et al., 2006a) this technique has been widely used especially to study spatial and temporal dynamics and to detect relevant compositional differences between communities. The

limitations of culture-dependent strategies have also been extensively discussed (Laiz et al., 2003; Tamaki et al., 2005) and we are aware that the cultivated strains may represent only a small and biased fraction of the total diversity.

For example the high proportion of *Gammaproteobacteria* obtained in this study can be in part biased, since strains able to rapidly grow on agar media may have been favored by the applied methodology.

In fact, culture-independent studies previously conducted reported the dominance of *Alphaproteobacteria* and *Bacteroidetes* on water samples from *Ria de Aveiro* (Henriques et al., 2004; Henriques et al., 2006a). This last phylum was particularly underrepresented within our culture collections suggesting the occurrence of *Bacteroidetes* refractory to cultivation in this environment.

3.4.2 Bacterioneuston vs. Bacterioplankton

Results obtained from both culture-dependent and culture-independent approaches revealed similar (although not identical) bacterial communities inhabiting the SML and UW in *Ria de Aveiro*. These results are in agreement with previous studies conducted in the Mediterranean Sea (Agogué et al., 2005a) and in the South Pacific Ocean (Obernosterer et al., 2008).

On the other hand, marked differences between both communities were previously observed for other geographical locations (e.g. North Sea and the Blyth estuary) but only when SML samples were collected using polycarbonate membranes (Franklin et al., 2005; Cunliffe et al., 2008; Cunliffe et al., 2009a).

The glass plate method here applied has been previously considered appropriate for sampling culturable and total bacteria from the SML and allegedly avoids biases imposed by selective adsorption properties of membranes (Agogué et al., 2004).

However, recently, Cunliffe and coworkers (2009a) argued that samples collected using this method may contain subsurface water in addition to the surface microlayer. Taking this into consideration, divergence between *Ria de Aveiro* SML and UW communities may be higher than noticed during this study and the detected differences are probably more pronounced than suggested by our results.

In fact, some consistent differences were detected. Abundance of heterotrophic bacteria was normally higher in the SML as previously described for other aquatic systems (Sieburth et al., 1976; Hardy, 1982). CFU enrichment in the SML has properly been related to higher nutrient concentrations and also to transportation of living cells from underlying waters via electrostatic interactions with rising particles (Joux et al., 2006).

In terms of composition, specific populations, detected as distinct DGGE band positions, were found and sequenced from the SML samples. Previous studies have also noticed structural differences (pronounced or minor) between SML and UW communities when using culture-independent fingerprinting techniques (Agogué et al., 2005a; Cunliffe et al., 2008).

The fact that most of the SML-specific bands retrieved during this study shared low level similarity with previously reported sequences may suggest the existence of estuarine SML-specific populations. However this aspect certainly needs further detailed investigation.

Regarding culturable heterotrophic bacteria, strong evidences suggesting the occurrence of unusual neustonic phylotypes absent from underlying waters were not gathered. In fact, the foremost difference detected was a higher abundance in UW of the *Actinobacteria* genera *Agrococcus* and *Kocuria*.

Also the genus *Vibrio* was mostly retrieved from the UW samples. Our results are in disagreement with previously reported studies which described *Actinobacteria* as more abundant in the surface microlayer (Agogué et al., 2005a) and the genus *Vibrio* as dominant in the bacterioneuston (Franklin et al., 2005).

As for other aspects, these contradictory results may be related to different sampling methodologies, geographical locations or even short-term spatial and temporal variabilities.

Differences between bacterioneuston and bacterioplankton in *Ria de Aveiro* were previously reported in what concerns the effects of ultraviolet radiation on both communities (Santos et al., 2011a; Santos et al., 2011b).

3.4.3 Biofilm-forming bacteria

The gelatinous nature of SML may promote the colonization by biofilm-forming bacteria (Cunliffe & Murrell, 2009). To our knowledge, this study presents the first attempt to detect differences between bacterioneuston and bacterioplankton focusing on bacteria known to form biofilms. For this we intentionally enriched our culture collection with members of *Pseudomonas* and *Aeromonas* and we also used a culture-independent assay specific for *Aeromonas*.

Both genera are ubiquitous in aquatic environments and some species/strains are known to form biofilms in water (Doğruöz et al., 2009). Consistent differences between SML and UW aeromonads communities were not identified neither by the analysis of the culture collections nor by the analysis of DGGE fingerprints.

In fact, DGGE fingerprints from both layers shared low similarity but apparently the observed variability cannot be explained by any of the considered variables (layer, site or light regimen).

In terms of *Pseudomonas,* an OTU (represented by isolate GCDN9-III in Table 3.1) was consistently overrepresented within SML samples (74% of the isolates). The ability of strains included in this OTU to form biofilms should be further investigated. Despite preliminary, our results suggest that differences confined to specific phylotypes could be relevant in distinguishing bacterioneuston and bacterioplankton. In accordance, additional efforts should be made to characterize the *Pseudomonas* communities in both layers.

3.4.4 Differences determined by spatial and temporal factors

The properties of SML may vary significantly along time and space (Peltzer et al., 1992; Santos et al., 2009). Therefore we hypothesized that the degree of similarity between adjacent bacterioneuston and bacterioplankton would differ between sampling sites and dates. For that reason, we sampled three geographical locations in four sampling dates and we also included samples collected during day and night. Clear diel patterns were not identified.

Even so, our results confirm that differences between both communities frequently vary according to spatial and temporal factors. In terms of culturable bacteria abundance,

differences were more pronounced in sampling site CN when compared with the other sampling locations.

Also, major differences were detected between samples collected during the third campaign. Water samples were not characterized in terms of organic matter content but site CN is mainly impacted by urban effluents, aquacultures and run-off from agriculture fields (Monteiro et al., 2007). Thus, it is possible that higher organic loads at this site may account for the differences observed.

In terms of composition, the strongest discrimination occurred between DGGE profiles derived from SML and UW samples collected from site CS and most of the SML-specific bands were retrieved from this site. The hydrocarbon contamination at this site (Coelho et al., 2010), which is expected to accumulate at the SML, may favor the establishment of unusual specific phylotypes.

Also, the spatial distribution of genera accounting for the most pronounced differences between both communities (*Agrococcus*, *Kocuria* and *Vibrio*) was unequal. *Agrococcus* and *Kocuria* were most frequently retrieved from site CS (71% of the isolates included in these genera) and *Vibrio* was mainly found at site CC (67% of the isolates).

Since temperature values were very stable between sites or sampling dates (Table 3.2) we can assume that this parameter was not a main driver of bacterioneuston and bacterioplankton compositional shifts. As for salinity, the higher values were observed in campaigns C3 and C4 (corresponding to a dry period). As previously described (Henriques et al., 2006a), this factor accounts for a considerable part of the variability in bacterial assemblages in the estuary but if it affects differently bacterioneuston and bacterioplankton remains to be clarified.

Our results suggest that differences between bacterioneuston and bacterioplankton are probably irregular and depend on temporal and spatial factors. This topic has been poorly addressed in previous studies and certainly warrants future investigation.

4. Molecular analysis of the diversity of genus *Psychrobacter* present within a temperate estuary

4.1 Introduction

Cold-adapted bacteria are often described as endemic organisms of extremely cold regions and its occurrence surpassing these environments is presumably sporadic (Martiny et al., 2006). However, 'Omics' technologies have revealed that psychrophilic bacteria harbour adaptive traits that could promote their dispersion beyond low-temperature locations (Methé et al., 2005; Cacace et al., 2010). For instance, the study of the transcriptome of the cold-adapted genus *Psychrobacter* revealed adaptations to significant temperature variations (Rodrigues et al., 2008; Bergholz et al., 2009). Even so, based on quantitative PCR and 16S rRNA gene libraries, *Psychrobacter* populations from extremely cold habitats were considerably more abundant and diverse than *Psychrobacter* populations retrieved from warmer regions (Rodrigues et al., 2009).

Global warming is reducing the extent of cold habitats thus affecting the evolution of cold-adapted bacteria. The extreme biotechnological relevance of cold-adapted bacteria as well as their fundamental role in biogeochemical cycles (Feller & Gerday, 2003) justifies the need to understand to what extent this bacteria can adapt to environmental warming. Studying the diversity of cold-adapted bacteria in temperate habitats will add knowledge on this topic.

The genus *Psychrobacter* includes Gram-negative coccobacilli that are non-pigmented, oxidase-positive, non-motile, psychrophilic or psychrotolerant, and halotolerant (Juni & Heym, 1986). At the time of writing, this genus included 34 species with validly published names. Most of the species described so far have been isolated from cold environments, including Arctic and Antarctic sea ice, water, soils and sediments (Bowman et al., 1996; Bowman et al., 1997; Yumoto et al., 2003; Romanenko et al., 2004; Shivaji et al., 2004; Bakermans et al., 2006). Less frequently new species have been isolated from temperate marine environments (Yoon et al., 2005b; Yumoto et al., 2010;) and other sources such as pigeon feces, food products, lung tissue and human blood (Vela et al., 2003; Yoon et al., 2003; Yoon et al., 2005a; Yassin & Busse 2009; Wirth et al., 2012).

Besides temperature, the occurrence of *Psychrobacter* was significantly associated with other environmental factors such as pH closer to neutrality, high salinity and higher concentrations of potassium and magnesium (Rodrigues et al., 2009). Anthropogenic-related factors may also influence the distribution of *Psychrobacter*. For

example, *Psychrobacter* enrichment has been noted in aquatic environments contaminated with hydrocarbons (Harwati et al., 2007; Prabagaran et al., 2007; Lo Giudice et al., 2010).

During a previous study based on culture-dependent methods, the genus *Psychrobacter* was, unexpectedly, the most frequently retrieved from the sea-surface microlayer (SML) and the underlying water (UW) of a temperate estuary (*Ria de Aveiro*, Portugal) during a warm season (May to July) (Azevedo et al., 2012). SML refers to the thin (1-1000 µm) film that forms between the hydrosphere and atmosphere (Azevedo et al., 2012).

To our knowledge, broad studies on the diversity of *Psychrobacter* within temperate aquatic environments have never been conducted. To provide a comprehensive picture of the diversity of *Psychrobacter* populations inhabiting SML and UW in *Ria de Aveiro*, culture-independent methods (e.g. DGGE and 16S rRNA clone libraries) specifically targeting this genus were applied. Also a collection of *Psychrobacter* isolates was analysed by molecular typing and 16S rDNA-based phylogenetic analysis.

4.2 Material and methods

4.2.1 Sampling

Ria de Aveiro is a shallow estuary on the north-west coast of Portugal (40º38'N, 8"45'W), about 45 km long and 8.5 km wide (Figure 5.1). This study was conducted during the warm season in 3 campaigns in May (C1), June (C2) and July (C3) and samples were retrieved from the SML and UW at three sites: *Cais do Chegado* (CC), *Costa Nova* (CN), and *Cais do Sporting* (CS). Water samples were retrieved as previously described (Azevedo et al., 2012). Briefly samples from SML were collected by adherence to glass and acrylic plates, and UW samples were collected by submerging a sterilized brown glass bottle and opening it at a depth of approximately 0.4 m. Samples were kept in cold and dark conditions during transport and were processed within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany) and measured using the Practical Salinity Scale.

4.2.2 Culture-dependent analysis of *Psychrobacter* populations

4.2.2.1 *Psychrobacter* isolates

Isolates were obtained as described by Azevedo and co-workers (2012). In summary, isolates were retrieved from Estuarine Agar (EA; Weiner et al., 1980) and GSP (Glutamate Starch Phenol Red Agar, *Pseudomonas/Aeromonas* selective agar) plates. DNA was purified as previously described (Azevedo et al., 2012). Eighty one isolates (42 from SML and 39 from UW samples) were affiliated to *Psychrobacter* by sequencing the 16S rRNA gene. The pure cultures were maintained on estuarine agar at 4ºC (after growing at the same culture media during 2 days at 30ºC) and as 20% (v/v) glycerol suspensions at -80ºC.

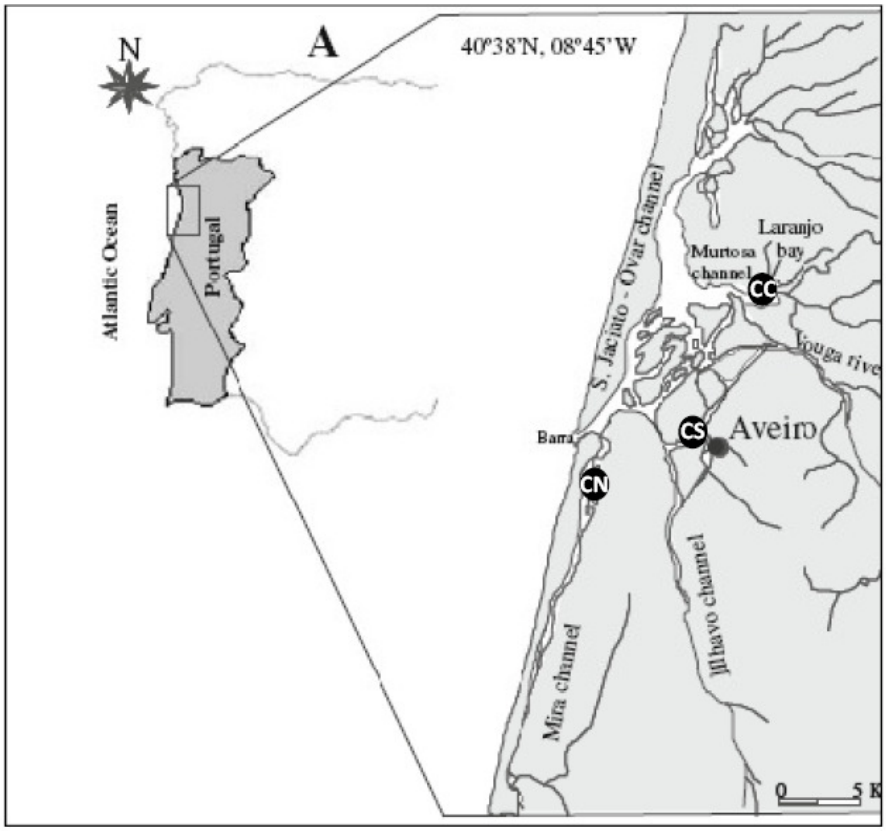

Figure 4.1 Map of *Ria de Aveiro* showing the location of the sampling sites *Costa Nova* (CN), *Cais do Sporting* (CS) and *Cais do Chegado* (CC).

4.2.2.2 REP-PCR genomic fingerprinting

All isolates were typed by using a REP-PCR method with primers REP-1R and REP-2I as described previously (Versalovic et al., 1991). PCR reactions were carried out with Taq polymerase, nucleotides and buffers from Promega. The PCR reaction mixtures (25µl) contained 1X PCR buffer, 200 µM of each nucleotide, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 7.5 pmol of each primer, 1U of Taq polymerase and 50–100 ng purified DNA. Amplification was carried out as follows: initial denaturation for 7 minutes at 95ºC, followed by 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 40ºC and extension for 8 min at 65°C and by a final extension of 16 min at 65ºC. The products were electrophoresed for 90 min under a constant voltage of 80V on a 1.5% (w/v) agarose gel containing 0.5X TAE (20 mmol/L Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na_2EDTA) and DNA markers purchased from MBI Fermentas (Vilnius, Lithuania). The gel images were acquired using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA USA) and analysed using the software package GelCompar 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

4.2.3 Cultured-independent analysis

4.2.3.1 DNA extraction and 16S rRNA gene amplification

For DNA extraction 200 mL water samples from SML and UW were filtered through 0.2-µm-pore-size polycarbonate filters (GE Water & Process Technologies). DNA purification was performed using the Genomic DNA Extraction Kit (MBI Fermentas) as described previously (Henriques et al., 2004).

Approximately 400bp of the 16S rRNA gene were amplified from water samples by using the *Psychrobacter*-specific primers 432-F/823-R and PCR conditions as described by Rodrigues et al. (2009). These and subsequent PCR reactions were carried out in a Bio-Rad MyCycler Thermal Cycler (Bio-Rad) using Taq polymerase, nucleotides and buffers purchased from MBI Fermentas.

4.2.3.2 Denaturing gradient gel electrophoresis (DGGE)

Psychrobacter-specific molecular fingerprints of each sample were obtained using a DGGE approach. PCR was as described above except that the following program was used: initial denaturation (94ºC for 5 min); 30 cycles of denaturation (92ºC for 30 s), annealing (59ºC for 30 s), and extension (72ºC for 30 s); and a final extension (72ºC for 30 min). Also, a GC clamp was attached to the 5' end of the forward primer in order to prevent complete melting of the PCR products during subsequent DGGE analysis (Muyzer et al., 1993).

PCR products were directly applied onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) in 0.5X TAE buffer (20 mmol/L Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na_2EDTA) with urea and formamide as denaturants. Linear denaturing gradient ranged from 35 to 62.5%. Electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad) at 60°C; initially a constant voltage of 20 V was applied for 15 min followed by 75 V during 16 hours. After electrophoresis, the gels were stained for 5 min with ethidium bromide and then rinsed for 20 min in distilled water. Gel images were acquired using the Gel Doc™ XR+ System (Bio-Rad).

DGGE profiles were analyzed using GelCompar II Software (Applied Maths). Cluster analysis of DGGE profiles was performed using the UPGMA method (group average method) applying Pearson correlation measure.

4.2.3.3 16S rRNA gene libraries

To construct *Psychrobacter*-specific 16S rRNA gene libraries, samples from the C3 campaign (July) were chosen. PCR products from SML and UW samples were mixed separately. The SML and UW mixed products were cloned using the TA cloning Kit according to manufacturer instructions (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). Libraries will be subsequently designated *Psysml* (obtained from SML samples) and *Psyuw* (from UW samples).

Inserts were amplified using vector-specific primers. PCR products with the expected size were purified with the Jetquick PCR Product Purification Spin Kit

(Genomed, Löhne, Germany). Sequencing reactions were carried out using the same primers by the company Stab-Vida (Oeiras, Portugal).

4.2.4 *Psychrobacter* population analysis

Taxonomic affiliation, alignment and clustering analysis were processed by using the pipeline available at Ribosomal Database Project (RDP-II) website (http://rdp.cme.msu.edu). OTUs (Operational Taxonomic OTUs) were defined based on a 99% cut-off value.

Classical indices to estimate richness (Chao's richness estimator) and diversity (Shannon index) were obtained from RDP-II pipeline for both clone libraries and also for the *Psychrobacter* culture collection. Distance matrixes were constructed and ∫-LIBSHUFF analysis was performed through MOTHUR (Schloss et al., 2009). The genetic variation within and among samples was estimated with an analysis of molecular variance (AMOVA) by using the program Arlequin version 3.1 (Excoffier et al., 2005).

One representative of each defined OTU was chosen to construct the phylogenetic tree within the MEGA 5.0 (Tamura et al., 2011) using the kimura-2 parameter and Neighbor-joining clustering method.

4.2.5 Nucleotide sequence accession numbers

Sequences were deposited in GenBank under the following accession numbers: JX897791 to JX897817 (for 16S rRNA gene clones) and JX897818 to JX897897 and JQ072080 (for *Psychrobacter* isolates).

4.3 Results and Discussion

4.3.1 Occurrence and characterization of *Psychrobacter* isolates from *Ria de Aveiro*

Psychrobacter isolates represented almost 25% of a culture collection of heterotrophic bacteria previously obtained from *Ria de Aveiro* (Azevedo et al., 2012). In the present study, these isolates (n=81) were categorized into 9 OTUs based on an identity criteria of 99% at the 16S rRNA gene sequence (Table 4.1). Almost 83% of the

isolates were included in the three dominant OTUs. All isolates were successfully typed by REP-PCR and 51 distinct profiles were obtained. Both the total number of OTUs and the number of REP profiles suggest the presence of a diverse population of *Psychrobacter* in the estuary. According to Bowman et al. (1997) and Vishnivetskaya et al. (2000) NaCl concentrations up to 1.0 M in the culture media and incubation at low temperatures can strongly select for *Psychrobacter* strains. However the abundance suggested by our results cannot be explained by culture conditions, since the ones applied during this study were not designed to enrich for *Psychrobacter*: samples were spread onto GSP and Estuarine Agar, which contain lower concentrations of NaCl, and plates were incubated at 22ºC.

The *Psychrobacter* isolates were retrieved from both water layers and the three sampling sites in all campaigns (Table 4.1).

Approximately the same number of isolates was retrieved from both layers. However, Shannon index values suggested higher diversity among the *Psychrobacter* population inhabiting SML (Table 4.1). SML communities are known to be subjected to a wider range of selective pressures than communities inhabiting UW. These pressures may be imposed by the presence of several contaminants, higher levels of UV radiation or higher temperature and salinity fluctuations (Maki, 1993; Cincinelli et al., 2005; Cuong et al., 2008; Azevedo et al., 2012): The persistence of culturable *Psychrobacter* phylotypes adapted to those pressures may account for the differences between water layers in terms of diversity. For example, hydrocarbons are known to be enriched in SML (Wurl & Obbard, 2004) and were reported to strongly impact the *Psychrobacter* communities (Lo Giudice et al., 2010).

Table 4.1 Diversity of *Psychrobacter* isolates retrieved from *Ria de Aveiro*.

OTU	Nº isolates (nº REP types)	Distribution by site			Distribution by water layer	
		CC	CN	CS	SML	UW
OTU1	17 (17)	2	1	14	10	7
OTU2	39 (20)	11	7	21	17	22
OTU3	11 (2)	1	4	6	9	2
OTU4	3 (3)	1	1	1	2	1
OTU5	5 (4)	0	1	4	1	4
OTU7	2 (2)	0	0	2	0	2
OTU9	2 (2)	0	0	2	2	0
OTU26	1 (1)	0	0	1	1	0
OTU27	1 (1)	1	0	0	1	0
Total		16	16	49	42	39
Shannon Index		1.04	1.49	1.47	1.54	1.36

Even so we are aware that this is an inference of the diversity based on the cultivable fraction and extrapolations to the community must be considered carefully.

About 60% of the *Psychrobacter* isolates were retrieved from sampling site CS (Table 4.1). This may also be related to higher contamination with hydrocarbons in this site (Coelho et al., 2010), reported to select for *Psychrobacter* spp. In fact, Prabagaran et al. (2007) showed that *Psychrobacter* representatives were strongly enriched due to addition of crude oil to seawater collected from off Ushuaia (Argentina), being undetectable through classic PCR approaches in the original samples. Also, *Psychrobacter* strains have emerged as hydrocarbon-degrading bacteria during studies conducted at Antarctic, Arctic and Indonesian seawaters (Gerdes et al., 2005; Harwati et al., 2007; Lo Giudice et al., 2010). The abundance of *Psychrobacter* in site CS may also be related to higher salinity values preferred by members of this genus (Romanenko et al., 2004; Ponder et al., 2005). Finally the *CS* site is located near a harbour that receives cod fishing boats coming from the North Sea, bringing salt-cured codfish. As previously reported, *Psychrobacter* was the dominant genus present in the cod skin mucus and survived to prolonged frozen storage and concentrations of NaCl of up to 25% (w/v) (Bjørkevoll et al., 2003). Thus we can speculate that runoff from cod-fishing boats can also contribute to the diversity and abundance of *Psychrobacter* in the CS site.

4.3.2 Dynamics of *Psychrobacter* populations

We developed and optimized a DGGE-based method to assess the structure and dynamics of *Psychrobacter* populations. The analysis included SML and UW samples from three sampling sites and three sampling periods (May to July).

DGGE fingerprints were obtained from all samples (Figure 4.2) and the method was highly reproducible (data not shown). In general profiles shared a high degree of similarity and the number of bands per profile was rather stable ranging from 10 to 13 bands. Clustering analysis grouped the obtained profiles preferentially according to sampling dates and sites rather than water layers (Figure 4.2). In fact, differences between SML and UW profiles were detected particularly in terms of the intensity of some bands (Figure 4.2).

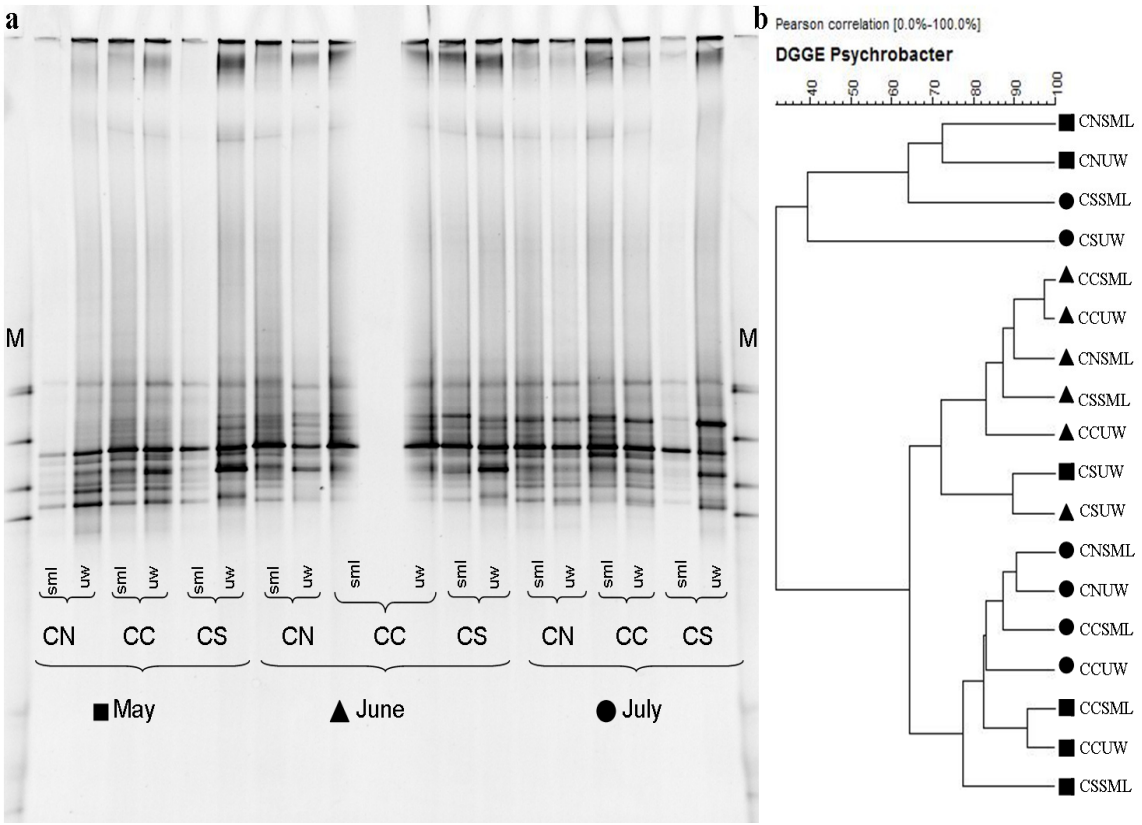

Figure 4.2 a) DGGE analysis of *Psychrobacter* populations in UW and SML samples collected in May ■, June ▲ and July ●. b) Dendrograms of DGGE patterns created using UPGMA method (group average method) applying Pearson correlation analysis.

DGGE results indicate that *Psychrobacter* populations were rather stable in the estuary suggesting the presence of several well-adapted phylotypes withstanding temporal and spatial environmental fluctuations.

4.3.3 Diversity of *Psychrobacter* populations

The diversity of the *Psychrobacter* community was further characterized by generating genus-specific *16S* rDNA libraries from samples collected in July. To assess differences between water layers, amplicons obtained from SML and UW samples were cloned separately.

A total of 106 clones were randomly picked and sequenced (51 from library *Psysml* and 55 from library *Psyuw*). All sequences affiliated with *Psychrobacter* members

confirming the specificity of the primers. Sequences were classified into 33 OTUs (at least 99% sequence similarity) (Figure 4.3). The number of sequences per OTU varied from only 1 to 25. Four OTUs included 47% of the total number of sequences. OTUs represented by only 1 sequence accounted for 45% of the total number of OTUs.

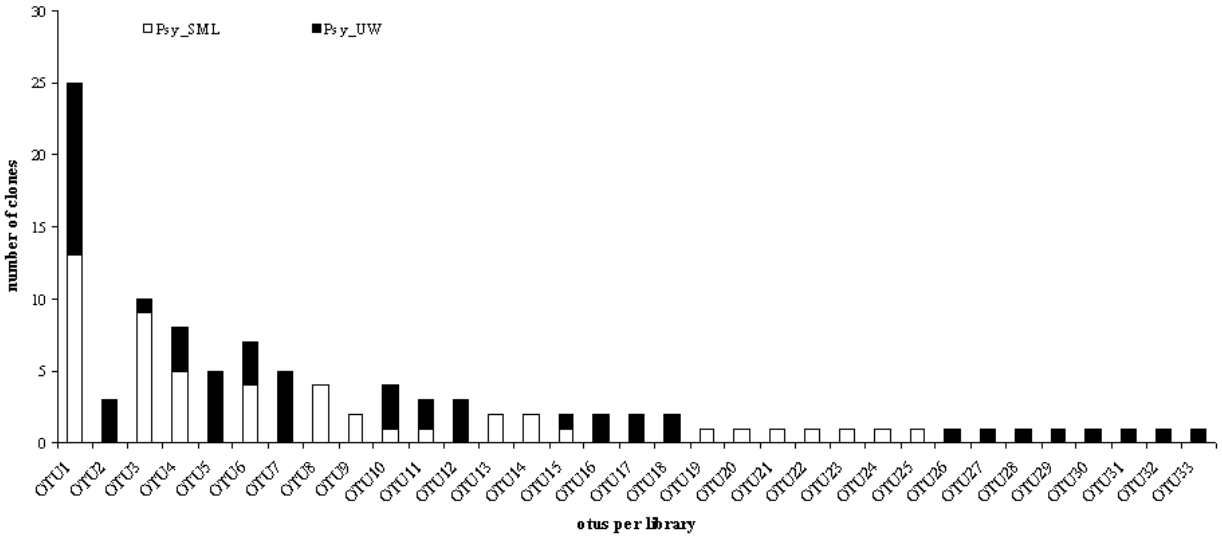

Figure 4.3 Relative abundance of the observed OTUs among *Psysml* and *Psyuw* clone libraries.

Sequences from the SML library were categorized in 18 OTUs and sequences from the UW library were distributed in 22 OTUs (Table 4.2). Only seven OTUs included sequences from both layers. Diversity and estimated richness were higher for the UW library. Also ∫-LIBSHUFF analysis indicate that *Psychrobacter* populations from SML and UW were significantly ($p<0.05$) different (Table 4.2). An AMOVA test was conducted to evaluate the variance in genetic diversity between *Psychrobacter* communities from SML and UW (Excoffier et al., 2005). According to the AMOVA results the *Psychrobacter* phylogenetic variance between layers was low (F_{ST}=7.22%) but significant ($p<0.001$). Genetic variation among populations probably results from different selective pressures occurring in SML and UW (Cunliffe et al., 2011). As previously referred SML is a place of accumulation of organic matter and of a variety of pollutants including hydrocarbons and heavy metals (Cincinelli et al., 2005; Cuong et al., 2008). Also stronger salinity and temperature variations are known to occur at this layer (Maki, 1993). Those factors may be determinant in selecting for different *Psychrobacter* phylotypes.

Table 4.2 Analysis of *Psychrobacter*-specific 16S rRNA gene libraries.

	Clone library	
	Psysml	*Psyuw*
Number of sequences	51	55
Number of OTUs	18	22
Shannon Index (H)	2.43	2.77
Chao1 estimator	27	30
% of coverage	66.7	70
$\int$-LIBSHUFF $_{(P\text{-value})}$ *	Psysml:Psyuw 0.0018	Psyuw:Psysml <0.0001

* *P-value* <0.05

Differences between SML and UW bacterial communities have been addressed in several aquatic systems including *Ria de Aveiro* (Azevedo et al., 2012). Although some controversy remains, different communities are presumed to occur in each layer (Cunliffe et al., 2011). However which phylogenetic groups contribute to those differences is mainly unknown. This study adds to the existent information by assessing differences at an intra-genus level.

4.3.4 Phylogenetic analysis of *Psychrobacter* isolates and clones

Phylogenetic analysis was done based on the partial 16S rRNA gene sequences (≈ 400bp) representing each OTU and the type strains of all *Psychrobacter* species described so far. Most sequences retrieved during this study fall into a large cluster that also included 13 *Psychrobacter* species mainly retrieved from marine or estuarine waters. *Psychrobacter* species from Arctic and Antarctic sediments as well as from fermented seafood, infected lung tissue, feces and human blood grouped in separate clusters.

According to the phylogenetic analysis a high diversity of *Psychrobacter* phylotypes were retrieved from *Ria de Aveiro* (Figure 4.4). These results suggest the existence of phylotypes adapted to temperate estuarine or marine aquatic environments. Salinity and contamination with hydrocarbons may play a role in the selection of these phylotypes (Bowman et al., 1997; Lo Giudice et al., 2010). Layer-specific clusters were not identified within the tree.

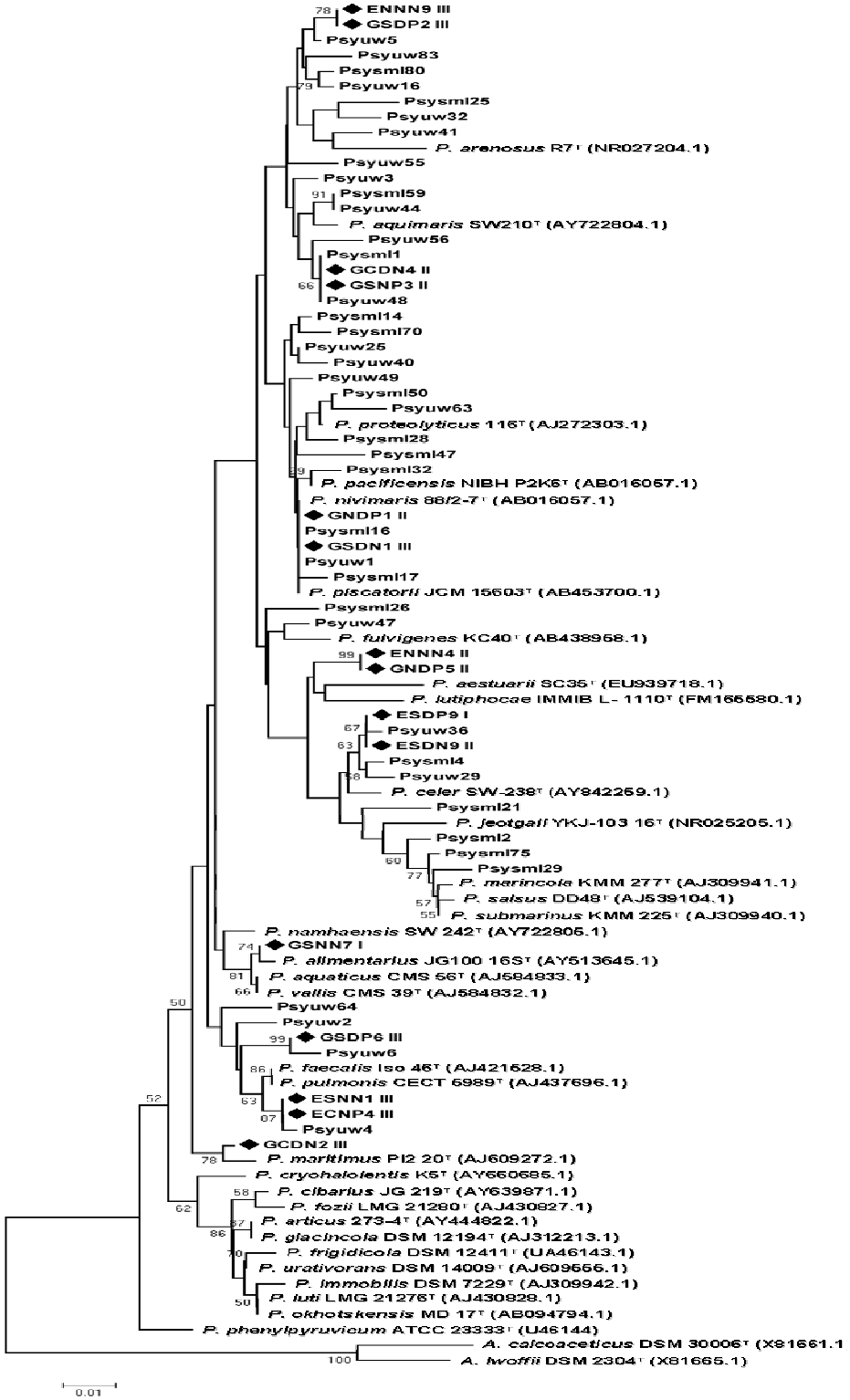

Figure 4.4 Phylogenetic tree based on 16S rRNA gene sequences of *Psychrobacter* strains (*Psycult*,♦) and clone libraries from SML (*Psysml*) and UW (*Psyuw*). One representative sequence of each *Psycult*, *Psysml* and *Psyuw* OTU was included. The phylogenetic tree was obtained by using the neighbor-joining method and rooted using 16S rRNA gene sequences from two *Acinetobacter* species. Bootstrap values higher than 50% are shown in nodes.

4.3.5 Final considerations

We conducted an in depth analysis of the structure and dynamics of the *Psychrobacter* populations in *Ria de Aveiro* by combining culture-dependent and culture-independent approaches. A new developed *Psychrobacter*-specific DGGE-based method was particularly helpful for the simultaneous analysis of a large number of samples allowing the clear identification of compositional shifts in the communities' structure. However, since DGGE only assesses the most dominant members of the community (Muyzer et al., 1993), clear differences between SML and UW communities were only observed through the analysis of 16S rDNA libraries, which provided a detailed picture of the phylogenetic composition of the *Psychrobacter* populations.

As often described by other authors (Kisand & Wikner, 2003; Brightwell et al., 2009) culture-dependent and culture-independent approaches did not provide overlapping results. A higher diversity was assessed using culture-independent methods and groups characterized by culturing were underrepresented when using culture-independent methods. Thus, the combination of the approaches here applied allowed obtaining a more comprehensive picture of *Psychrobacter* communities.

Our overall results revealed the presence in *Ria de Aveiro* of populations of *Psychrobacter* that are composed by a large diversity of members suggesting that this genus is well-adapted to this environment. Also, different *Psychrobacter* populations were found in the SML and UW. Observed diversity trends may be related with environmental factors such as salinity and/or anthropogenic pressures such as the presence of hydrocarbons.

5. Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine environment

5.1 Introduction

A large fraction of the Earth's surface is covered by water. The interface between hydrosphere and atmosphere is represented by a thin gelatinous surface film designated as sea-surface microlayer (SML), where many ecological processes take place (Liss & Duce, 1997).

The SML is distinct from underlying waters (UW) (Liss & Duce, 1997), since higher fluctuations in salinity and temperature occur and the exposure to ultra-violet radiation is also greater than in UW. Also, in the SML higher amounts of organic compounds (proteins, lipids and polysaccharides) and anthropogenic contaminants (hydrocarbons, metalloids, pesticides and antibiotic residues) accumulate (Cincinelli et al., 2005; Cuong et al., 2008; Cunliffe & Murrell, 2009). Moreover, even under greater selective pressures, the SML harbours a more abundant and active bacterial community, the bacterioneuston, comparing to underlying waters (Azevedo et al., 2012; Hardy, 1982; Sieburth et al., 1976).

The gelatinous nature of the SML combined with strong selective pressures and high bacterial densities stimulate the production of secondary metabolites (Maki, 2002). In fact, for example antibiotic producers have been frequently retrieved from the SML (Hakvåg et al., 2008).

The production of antimicrobials together with persistence of anthropogenic-derived antibiotic residues in the SML (Walczak & Donderski, 2004) may select for and foment the dissemination of antibiotic resistance genes (ARGs). In addition bacterial growth in biofilms, expected to occur in the SML, might also promote horizontal gene transfer and resistance to antimicrobial agents (Hermansson et al., 1987; Cunliffe & Murrell 2009; Oliveira et al., 2012). Thus, the SML may be a natural reservoir of antibiotic resistance genes and an incubator of new gene combinations.

However, most studies concerning antibiotic bacterial resistance in estuarine and coastal water systems focused on bulk water (Henriques et al., 2006c; Kümmerer, 2009; Figueira et al., 2011), and consequently the relevance of the SML in developing, selecting and spreading ARGs has been almost ignored.

Estuarine systems are strongly influenced by anthropogenic activities accumulating high levels of pollutants and often becoming eutrophicated (Keddy, 2000), and thus gathering ideal conditions for the establishment of a distinct bacterioneuston community.

In a previous study, we have focused our attention on the phylogenetic composition of the bacterioneuston and bacterioplankton communities from a highly polluted estuarine system (*Ria de Aveiro*, Portugal) (Azevedo et al., 2012). The aims of the present study are: 1) to compare the prevalence of cultivable antibiotic-resistant bacteria in the SML and UW in *Ria de Aveiro*; 2) to compare antibiotic resistance profiles between isolates previously obtained from SML and UW samples; 3) to assess and compare the occurrence and diversity of antibiotic resistance genes and integrons in the same isolates.

5.2 Material and methods

5.2.1 Heterotrophic plate counts on antibiotic-selective media

SML and UW samples were collected in three occasions (C1, C2 and C3) in 2008 at three sites named *Cais do Chegado* (CC), *Costa Nova* (CN), and *Cais do Sporting* (CS) within the estuary *Ria de Aveiro* as described by Azevedo and co-workers (2012). Briefly, SML samples (250 mL) represented by a water layer of 60-100 μm were collected with an acrylic or a glass plate (Azevedo et al., 2012). Underlying waters (1 L) were collected at approximately 0.4 m depth in 2 L autoclaved bottles.

From each water sample 100μl were directly spread onto EA and GSP plates without added antibiotics. Also 1 mL of each sample was serially diluted in 0.9% NaCl and 100μl of each dilution was spread in the same media to obtain colony counts between 30 and 300 per plate. Total heterotrophic plate counts (HPC) were determined using these plates.

Resistant bacteria were cultivated by the same spreading procedure in the same culture media supplemented with ampicillin (50μg/μl), tetracycline (20 μg/μl) and streptomycin (10 μg/μl). These concentrations are above the breakpoints defined by the Clinical and Laboratory Standards Institute (CLSI, 2005) for most phylogenetic groups and were chosen to select for highly resistant bacteria. Colony-forming units (CFU/ml) were counted in triplicate (three independent plates) after 4 days of incubation at 22ºC in the dark.

5.2.2 Bacterial strains

A total of 352 bacterial isolates were obtained from SML and UW within the estuary *Ria de Aveiro* in a previous work, corresponding to approximately 10 isolates selected from each sample (Azevedo et al., 2012). From those, 12 isolates were impossible to recover in successive cultures and were excluded from the current study. Isolates were typed using REP-PCR with primers REP-1R and REP-2I as described previously (Versalovic et al., 1991). PCR reactions were carried out with Taq polymerase, nucleotides and buffers from Promega (Madison, WI). The PCR reaction mixtures (25µl) contained 1X PCR buffer, 200 µM of each nucleotide, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 7.5 pmol of each primer, 1U of Taq polymerase and 50–100 ng purified DNA. Amplification was carried out as follows: initial denaturation for 7 minutes at 95ºC, followed by 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 40ºC and extension for 8 min at 65°C and by a final extension of 16 min at 65ºC. The products were electrophoresed for 90 min under a constant voltage of 80V on a 1.5% (w/v) agarose gel containing 0.5X TAE (20 mmol/L Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na_2EDTA) and DNA markers purchased from MBI Fermentas (Vilnius, Lithuania). The gel images were acquired using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA, USA) and analysed using the software package GelCompar 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). From 340 isolates, a total of 171 isolates displaying different profiles were identified based on the partial sequence of the 16S rRNA gene (Azevedo et al., 2012). Isolates (80 isolates from SML and 91 from UW) affiliated with 34 genera within 6 classes (Table 5.1) and were included in the present study.

5.2.3 DNA extraction

Isolates were cultured in Luria-Bertani agar plates (Merck, Germany) and were incubated at 30ºC overnight. The total DNA was obtained by ressuspending 2 isolated colonies in 100 µl of buffer B1 (50 mM Tris-Cl pH 8.0; 50 mM EDTA pH 8.0; 0.5% Tween 20; 0.5% Triton X-100) and 100µl of master-mix (1 ml of buffer B1; 2 mg/ml of Lysozyme; 4.5 mg/ml of Proteinase K: 7.5 U of RNAse A). Cell suspension was mixed vigorously and incubated for 30 minutes at 37ºC. Following, 70 µl of B2 (3 M

Guanidine Hydrochloride; 20% Tween 20) were added and the mixture was incubated for 30 minutes at 50ºC. Subsequently the DNA Extraction Kit (#K0513 – MBI, Fermentas, Vilnius, Lithuania) was used according to the manufacturer instructions.

5.2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the agar disk diffusion method as recommended by the CLSI. Isolates representing different REP profiles (n=171) were tested for susceptibility to 9 antimicrobial agents representing six classes of antibiotics: (1) β-lactams - ampicillin (10µg), imipenem (10µg), cephalothin (30µg); (2) aminoglycoside - streptomycin (10µg), gentamicin (10µg); (3) phenicols - chloramphenicol (30µg); (4) tetracyclines - tetracycline (30µg); (5) –the combination sulfamethoxazole/trimethoprim (25µg) and (6) quinolones - nalidixic acid (30µg). Disks were purchased from Oxoid (Hampshire, United Kingdom). Antimicrobial activities were determined as clear zones of inhibition around the antibiotic disks, after incubation at 30ºC for 24 h. Isolates were classified as sensitive, intermediate or resistant taking into account the CLSI guidelines (CLSI, 2005). Whenever genus-specific guidelines were not available guidelines for the Enterobacteriaceae were used for Gram-negative strains and guidelines for *Staphylococcus* spp. were used for Gram-positive strains. *Escherichia coli* ATCC 25922 was used as a quality control strain.

5.2.5 Antimicrobial resistance genes

All isolates were tested by PCR for the presence of genes conferring resistance to beta-lactams, tetracyclines, chloramphenicol, aminoglycoside, and sulphonamides by using primers and PCR conditions previously reported (Henriques et al., 2006c, 2008) (see also Table 5.2). Isolates carrying resistance genes were included in each PCR run as positive controls. The positive controls for amplification of the sulphonamide resistance genes *sul1*, *sul2* and *sul3* were as described previously (Henriques et al., 2006c; Correia et al., 2003). A PCR mixture with no DNA added was used as a negative control. Gel electrophoresis was performed on 1.5% agarose gel and stained with ethidium bromide.

Table 5.1 Summary of bacteria isolated from SML and UW samples.

	Phylogenetic group		Number of isolates	
	Class	**Genera**	**SML**	**UW**
Gram-positive	Actinobacteria	*Agrococcus*	0	6
		Arthrobacter	1	1
		Brevibacterium	0	1
		Corynebacterium	1	0
		Kocuria	0	2
		Microbacterium	0	1
		Micrococcus	1	0
		Unclassified Micrococcaceae	2	5
	Bacilli	*Aerococcus*	1	0
		Bacillus	3	1
		Exiguobacterium	0	1
		Planococcus	2	0
		Staphylococcus	1	0
Gram-negative	Alfaproteobacteria	*Brevundimonas*	0	3
		Erythrobacter	1	1
		Agrobacterium	1	0
		Pseudorhodobacter	2	2
	Betaproteobacteria	*Alcaligenes*	2	1
		Comamonas	1	0
		Delftia	0	1
	Gamaproteobacteria	*Aeromonas*	10	7
		Shewanella	6	8
		Pseudoalteromonas	0	1
		Rheinheimera	2	4
		Erwinia	2	0
		Halomonas	2	0
		Marinomonas	1	2
		Acinetobacter	5	8
		Pseudomonas	10	7
		Psychrobacter	15	13
		Vibrio	1	7
		Lysobacter	0	1
		Stenotrophomonas	2	1
		Unclassified Enterobacteriaceae	5	6

5.2.6 Detection and characterization of integrons

The presence of *IntI1*, *IntI2* and *IntI3* genes, encoding class 1, 2 and 3 integrases, was investigated by PCR as previously described and using the same positive controls (Moura et al., 2007) (see Table 5.2). All integrase-positive isolates were examined to determine the sizes of integrons variable regions. Primer set 5'CS/3'CS was used to amplify the class 1 integron gene cassette region according to what has been described elsewhere (Moura et al., 2007) (see Table 5.2). PCR products were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) and used as

template in the sequencing reaction carried out by the company GATC (Germany). When bands with different sizes were amplified, PCR products were cloned using a TA cloning kit (Invitrogen, California, USA) according to the manufacturer's instructions. Clones representative of different amplicons were sent for sequencing. Online similarity searches were performed using the BLAST software (Altschul et al., 1997).

5.2.7 Statistical analysis

Data retrieved from heterotrophic plate counts on antibiotic-selective media were analysed using t-test through PAST version 2.04 (http://palaeoelectronica.org/2001_2001/past/issue2001_2001.htm.) (Hammer et al., 2001).

5.2.8 Nucleotide sequence accession numbers

The nucleotide sequences were deposited in the GenBank nucleotide database under the following accession numbers: JX646699 to JX646702.

5.3 Results

5.3.1 Prevalence of antibiotic-resistant bacteria

The percentages of ampicillin-, streptomycin- and tetracycline-resistant cultivable bacteria found in the two water layers are shown in Table 5.3. In general, resistance to streptomycin was the most prevalent (5.86% to 42.87% of total HPC), followed by ampicillin (2.29% to 12.37%) and tetracycline (0.87% to 7.21%). Levels of resistance were significantly higher for the SML samples (p-value < 0.05) with only a few exceptions (resistance to tetracycline in the C2 and C3 campaigns). The prevalence of ampicillin-resistant bacteria in the SML was 3 to 4 times higher than in UW and streptomycin-resistant bacteria were 2 to 3 times more prevalent in the SML. For tetracycline, significant differences were only detected in the first campaign (5 times more prevalent in the SML).

Table 5.2 Primers and PCR conditions

Primer pair	Target	Sequence (5' – 3')	Anealing temperature (ºC)	Amplicon size (bp)	Control strain	Reference
TEM_F TEM_R	*bla*$_{TEM}$	AAAGATGCTGAAGATCA TTTGGTATGGCTTCATTC	44	425	*Klebsiella pneumoniae* 6T [a]	[a] Henriques et al., 2006b
SHV_F SHV_R	*bla*$_{SHV}$	GCGAAAGCCAGCTGTCGGGC GATTGGCGGCGCTGTTATCGC	62	304	*K. pneumoniae* 2s [a]	
OXA-B_F OXA-B_R	*bla*$_{OXA-B}$	CAAGCCAAAGGCACGATAGTTG CTCAACCCATCCTACCCACC	56	561	*Aeromonas* sp. G.N1.15 [a]	
IMPF IMPR	*bla*$_{IMP}$	GAATAGAGTGGATTAATTCTC GGTTTAAYAAAACAACCACC	55	232	*K. pneumoniae* KP99c196 [a]	
TetAF TetAR	*tetA*	GCTACATCCTGCTTGCCTTC GCATAGATCGCCGTGAAGAG	53	211	*Escherichia coli* M.I10.34 [b]	[b] Henriques et al., 2008
TetBF TetBR	*tetB*	TCATTGCCGATACCACCTCAG CCAACCATCATGCTATTCCATCC	53	391	*E. coli* M.N1.616 [b]	
TetEF TetER	*tetE*	ATGAACCGCACTGTGATGATG ACCGACCATTACGCCATCC	53	744	*Aeromonas* sp. G.I10.2 [b]	
TetMF TetMR	*tetM*	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	55	406	*E. coli* M.I10.34 [b]	
aadA1_F aadA1_R	*aadA1*	TATCAGAGGTAGTTGGCGTCAT AATGAAACCTTAACGCTATGGAAC	54	485	*Aeromonas* sp. G.N1.15 [a]	[a] Henriques et al., 2006b
catF catR	*cat*	CCTGCCACTCATCGCAGT CCACCGTTGATATATCCC	55	623	*Aeromonas* sp. G.N1.15 [a]	
sulF1 sulR1	*sul1*	CTGAACGATATCCAAGGATTYCC AAAAATCCCATCCCCGGRTC	50	239	*Escherichia coli* M.I10.40 [a]	
sul2-F sul2-R	*sul2*	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	69	293	*Escherichia coli* M.I10.40 [a]	
sul3F sul3R	*sul3*	AAGAAGCCCATACCCGGRTC ATTAATGATATTCAAGGTTTYCC	50	236	*K. pneumoniae* (intI3+) [c]	[c] Moura et al., 2007
intI1F intI1R	*Class 1 integrase gene*	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	55	280	*Salmonella enterica* ser Typhimurium (intI1+) [c]	
intI2F intI2R	*Class 2 integrase gene*	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	52	233	*E. coli* (intI2+) [c]	
intI3F intI3R	*Class 3 integrase gene*	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	50	600	*K. pneumoniae* (intI3+) [c]	
5'-CS 3'-CS	*class 1 integron variable region*	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	58.5	variable	*Aeromonas* sp. G.N1.15 [a]	[a] Henriques et al., 2006b

Table 5.3 Prevalence of Antibiotic-resistant bacteria in SML and UW

Campaign	Sample	Total of HPC[a] (CFU/ml)	% of HPC resistant to:		
			Streptomycin	Tetracycline	Ampicillin
C1	SML	2.9×10^4	34.40 ± 2.24	7.21 ± 0.33	12.37 ± 1.23
	UW	6.2×10^3	21.33 ± 1.37	1.49 ± 0.25	3.20 ± 0.74
C2	SML	1.3×10^4	17.60 ± 2.11	0.87 ± 0.60	10.11 ± 1.04
	UW	1.8×10^4	5.86 ± 2.66	0.99 ± 1.90	3.99 ± 1.82
C3	SML	3.6×10^4	42.87 ± 6.67	0.91 ± 0.33	7.73 ± 0.45
	UW	1.9×10^4	12.53 ± 0.71	1.17 ± 1.01	2.29 ± 0.60

[a]Heterotrophic plate count

5.3.2 Antibiotic susceptibility testing

Susceptibility to all tested antibiotics was observed in 36% (61 out of 171) of the isolates. Predominant resistances were observed to cephalothin (46% of the isolates were resistant to this antibiotic), followed by ampicillin (40%) and streptomycin (34%). Isolates were most frequently susceptible to imipenem (only 6% of the isolates were resistant to this antibiotic), tetracycline (4%) and gentamicin (3%). Significant differences between isolates collected from SML and UW were not observed (Figure 5.1).

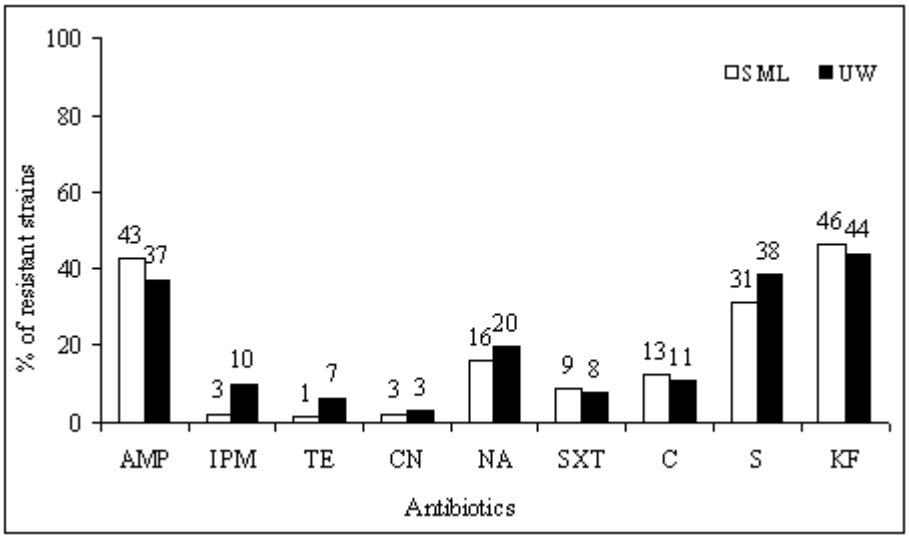

Figure 5.1 Percentages of strains obtained from the SML and UW that were resistant to each of the antibiotic tested. Antibiotic abbreviations: AMP, ampicillin; IMP, imipenem; TE, tetracycline; CN, gentamicin; NA, nalidixic acid; SXT, sulfamethoxazole/trimethoprim; C, chloramphenicol; S, streptomycin; KF, cephalothin.

Since antibiotic resistance profiles may be dependent on the phylogenetic groups, results were separately analysed for the most commonly retrieved genera (*Aeromonas*, *Acinetobacter*, *Pseudomonas*, *Psychrobacter* and *Shewanella*) as well as for the isolates classified as Enterobacteriaceae (Table 5.4). Most groups were highly resistant (resistance rates > 30%) to ampicillin, cephalothin and streptomycin. Lower levels of resistance to these antibiotics were only registered for *Acinetobacter* against streptomycin and for *Shewanella* against ampicillin. Additionally *Psychrobacter* members were exceptionally sensitive (resistance rates < 18%) to all antibiotics tested. All groups were highly susceptible (resistance rates < 18%) to imipenem, gentamicin and tetracycline. Resistance levels to chloramphenicol, nalidixic acid and trimethoprim/sulfamethoxazole were generally low with a few exceptions: *Acinetobacter* and *Pseudomonas* were highly resistant to trimethoprim/sulfamethoxazole and *Pseudomonas* members were also highly resistant to chloramphenicol and nalidixic acid.

The overall frequency of multiresistant isolates (resistant to 3 or more classes of antibiotics) was 14% (24 isolates out of a total of 171 isolates). In terms of multiresistance, results obtained for SML and UW isolates were also similar (Figure 5.2). For multiresistant isolates, 16 antibiotic resistance patterns were observed (Table 5.5). The most common resistance profile included resistance to ampicillin, nalidixic acid, streptomycin, cephalothin, chloramphenicol and trimethoprim sulfamethoxazole (2 isolates from SML and 2 from UW). The comparison between phylogenetic groups revealed that multiresistance was more frequent among *Pseudomonas* strains (Table 5.4).

Table 5.4 Antibiotic resistance rates of the most frequently retrieved genera and Enterobacteriaceae.

	Resistance to Antibiotics (%)									
Phylogenetic group	Ampicillin	Cephalothin	Imipenem	Tetracycline	Chloramphenicol	Gentamicin	Nalidixic acid	Streptomycin	Trimethoprim / sulfamethoxazole	MDR[a]
Acinetobacter (n=13)	53.8	92.3	7.7	7.7	15.4	0.0	15.4	7.7	38.5	15.4
Aeromonas (n=17)	100	82.2	5.9	17.6	0.0	0.0	17.5	88.2	0.0	23.5
Enterobacteriaceae (n=13)	46.2	30.8	0.0	7.7	7.7	0.0	7.7	30.8	7.7	15.4
Pseudomonas (n=17)	82.4	88.2	0.0	5.9	47.1	0.0	47.1	41.2	47.1	52.9
Psychrobacter (n=28)	3.6	3.6	7.1	0.0	0.0	0.0	10.7	17.9	0.0	0.0
Shewanella (n=14)	21.4	100	7.1	0	0.0	7.1	14.3	42.9	7.1	14.3

5.3.3 Screening for antibiotic resistance and integrase genes

The presence of genes conferring resistance to β-lactams, tetracyclines, chloramphenicol, aminoglycoside, and sulphonamides was analysed by PCR in all the 171 isolates (Table 5.2). Genes *bla*$_{TEM}$, *bla*$_{OXA-B}$, *bla*$_{SHV}$, *bla*$_{IMP}$, *tet*(A), *tet*(B), *tet*(E), *tet*(M), *cat*, *sul1*, *sul2*, *sul3* and *aad*A were chosen as they have been the most frequently detected acquired genes both in clinical and environmental isolates. Surprisingly, despite the considerable high levels of resistance against β-lactams and aminoglycosides, genes conferring resistance to these antibiotics were not detected. Genes conferring resistance to chloramphenicol were also not found. Among 7 isolates displaying resistance to tetracycline, *tet*(E) was detected in two isolates belonging to genus *Aeromonas* and *tet*(M) was detected in one *Pseudomonas* isolate.The gene *sul1* was amplified from 3 isolates belonging to genus *Aeromonas* and *sul2* was detected in one *Pseudomonas* isolate.

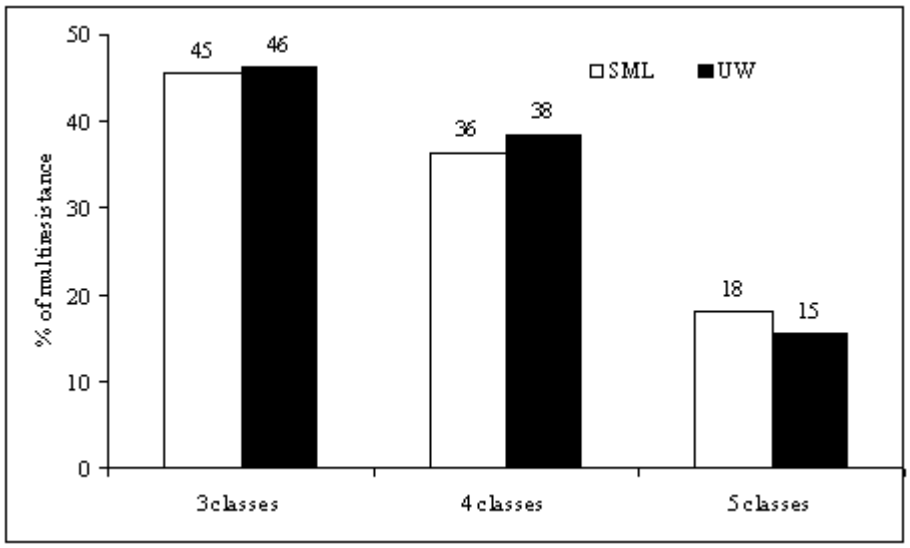

Figure 5.2 The percentage of multiple antibiotic-resistant bacteria from the SML (bacterioneuston) and UW (bacterioplankton).

The *intI1* gene was present in 2.11% of the isolates, all affiliated with *Aeromonas*, while genes *intI2* and *intI3* were not detected in any isolate. The variable region of class 1 integrons was successfully amplified with the primers 5'CS/3'CS. Sequencing analysis

revealed 4 different arrays: *aadA1*, *aadA13*, *catB8* and *catB8-aadA1*. The last two arrays were amplified from the same isolate.

Table 5.5 Antibiotic resistance patterns of SML and UW isolates

Antibiotic resistance patterns	Nº of multi-resistant isolates (*n=24*)	
	SML	UW
AMP, TE, C[b]	0	1
AMP, NA, S[a]	2	1
TE, C, KF[b]	0	1
NA, S, KF[b]	0	1
AMP, NA, SXT, S[b]	0	1
AMP, NA, SXT, KF[c]	1	0
AMP, NA, S, KF[a]	1	1
AMP, C, S, KF[b]	0	1
AMP, TE, NA, S, KF[b]	0	3
AMP, TE, NA, SXT, KF[c]	1	0
AMP, NA, SXT, C, KF[c]	1	0
AMP, NA, C, S, KF[c]	1	0
IMP, CN, NA, SXT, S, KF[b]	0	1
AMP, IMP, CN, C, S, KF[c]	1	0
AMP, IMP, SXT, C, S, KF[c]	1	0
AMP, NA, SXT, C, S, KF[a]	2	2

[a]The most common pattern of multiple-drug resistance among both water collections. Antibiotic resistance patterns specific of [b]UW and [c]SML isolates. AMP, ampicillin; KF, cephalothin; IMP, imipenem; TE, tetracycline; C, chloramphenicol; CN, gentamicin; NA, nalidixic acid; S, streptomycin; SXT, sulfamethoxazole/trimethoprim.

5.4 Discussion

We compared the persistence of antibiotic resistance among cultivable bacteria from the SML and UW in an estuarine system. We hypothesized that SML would be a reservoir of antibiotic resistance.

Specifically we compared the prevalence of tetracycline-, ampicillin- and streptomycin-resistant bacteria between both water layers. These antibiotics have been used for long not only in human medicine but also in agriculture, aquaculture and veterinary (Chelossi et al., 2003). Accordingly resistance to these drugs is highly disseminated in several environments (Olaniran et al., 2009; Barkovskii & Bridges, 2011). Results obtained during this study showed significant differences between SML and UW in terms of prevalence of resistance to all tested antibiotics. Differences may arise from the fact that SML accumulates pollutants including antibiotics (Hermansson et al., 1987;

Walczak & Donderski, 2004) and antibiotic concentrations in the environment are the main factor contributing to the selection of resistant bacteria (Kümmerer, 2004). In addition to the selective pressure exerted by antibiotics themselves, other compounds such as heavy metals, disinfectants or pesticides also contribute to the maintenance of antibiotic resistance (Jones et al., 1991). SML has been described to accumulate nickel, copper, arsenic, chlorinated pesticides and polychlorinated biphenyls (Cuong et al., 2008; Wurl & Obbard, 2005). On the other hand producers of antimicrobial compounds have often been described among particle-attached bacteria rather than free-living bacteria (Hakvåg et al., 2008; Wilson et al., 2010, 2011). Particle-attached bacteria may be enriched in SML due to accumulation of higher amounts of organic particles in this water layer (Cunliffe & Murrell, 2009).

Besides determining the prevalence of antibiotic-resistant bacteria in both layers using antibiotic-supplemented agar, during this study we also compared antibiotic resistance profiles of isolates from SML and UW. Isolates were obtained in non-selective agar without antibiotic. No significant differences in terms of resistance profiles or prevalence of multiresistance were detected between SML and UW isolates. These apparently contradictory results are probably due to the fact that generally resistant bacteria were a minor component of the SML and UW bacterial communities preventing the detection of differences between culture collections. In fact antibiotic resistance levels among our bacterial collections were generally low and multiresistance was infrequent. For example members of the genus *Psychrobacter* were highly sensitive to all antibiotics tested. *Psychrobacter* was the most frequently retrieved genus representing almost 17% of the total number of isolates. Although other studies reported considerable higher resistance levels in estuarine bacteria (Zheng et al., 2011), those studies were either focused on resistant bacteria selected on antibiotic-supplemented agar (Evangelista-Barreto et al., 2010) or on specific bacterial groups known for their high resistance levels to several antibiotics (Henriques et al., 2006c). When unbiased selections were conducted results were similar to the ones obtained during this study (Mudryk, 2004; Mudryk & Skorczewski, 2009).

Comparative information on antibiotic resistance between SML and UW is still scarce. Even so, a few studies reported differences between SML and UW in terms of prevalence

of resistant bacteria (Hermansson et al., 1987; Jones et al., 1991). Those studies were also based on counting antibiotic-resistant bacteria in antibiotic-supplemented agar.

In this study colonies were randomly selected from petri dishes, which may have excluded some of the less common members of the community. Even so this procedure was adopted since colony selection based on morphological traits commonly results in overrepresentation of these less prevalent members. Also the representativeness of our culture collection was improved by collecting a high number of samples and by using two different culture media.

We assessed the presence and diversity of acquired ARGs commonly detected both in clinical and environmental isolates. As previously described antibiotic resistance phenotypes and genotypes may not match (Henriques et al., 2006c). Thus, we decided to screen for the presence of resistance genes in all the isolates independently of their resistance phenotypes. The presence of the antibiotic resistance genes in isolates with a susceptible phenotype is worth evaluating since in combination with other resistance mechanisms or in a different genomic context these genes may confer or potentiate resistance.

Most of the times we failed to detect any of the inspected genes even in resistant isolates. For example the presence of the common acquired *bla* genes encoding beta-lactamases was not demonstrated in any of the isolates, even when an ampicillin resistance phenotype was detected. Again, our results may be due to the fact that an unbiased selection was conducted. This strategy resulted in a collection of isolates belonging to a wide range of genera, for some of which antibiotic resistance mechanisms had never been characterized. These mechanisms may be intrinsic rather than the acquired ones inspected during this study. For example, trends of antibiotic resistance were essentially the same for the genera most commonly retrieved in our study and included high levels of resistance to ampicillin, cephalothin and streptomycin. Intrinsic antibiotic resistance mechanisms to β-lactams have already been described such as the production of chromosomal-encoded β-lactamases in *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Vibrio*, *Stenotrophomonas* or *Shewanella* (Livermore, 1995). Additionally *Acinetobacter*, *Pseudomonas* and *Aeromonas* are noted for their intrinsic resistance to antibiotics due to several other mechanisms such as the production of amynoglicoside-modifying enzymes, the diminished expression of outer membrane proteins and the over expression of efflux pumps (Bonomo & Szabo,

2006; Janda & Abbott, 2010). For these genera the combination of several resistance mechanisms often occurs leading to the emergence of multiresistance strains (Bonomo & Szabo, 2006; Evangelista-Barreto et al., 2010). In our study multiresistance was particularly associated to *Pseudomonas* and *Aeromonas*.

Concerning tetracyclines, the genes *tet*(E) and *tet*(M) were detected in *Aeromonas* and *Pseudomonas* respectively. *tet*(E) has previously been described has the most frequent tetracycline resistance mechanism in *Aeromonas* (Henriques et al., 2008) and *tet*(M) is also common among Gram-negative isolates including *Pseudomonas* (Brown et al., 2008).

The prevalence of integrons was also assessed and class 1 integrons were detected in 2.11% of our isolates. This prevalence is comparable to what has been reported for estuarine bacteria from other geographical locations (Rosser & Young, 1999). All class 1 integrons were detected in *Aeromonas* isolates (2 from SML and 1 from UW samples), predominantly carrying the *aadA*-type cassettes (*aadA1*, *aadA13*), conferring resistance to streptomycin. These cassettes are reported as the most frequent gene cassettes in bacterial isolates (Moura et al., 2009) and have been frequently detected in *Aeromonas* (Henriques et al., 2006c; Moura et al., 2007; Laroche et al., 2009). Results obtained during this study must be interpreted having in mind that only the culturable fraction of the bacterial community was considered. We are aware that only a minor fraction of the environmental bacterial community can be cultivated under laboratory conditions (Azevedo et al., 2012). However, the large majority of the studies on antibiotic resistance conducted so far are based on the characterization of pure cultures. The main reasons are related to the current limitations of culture-independent methodologies (Henriques et al., 2006b). On one hand fragments amplified from total DNA are not necessarily indicative of the presence of functional genes. On the other hand the hosts of those genes usually cannot be identified by culture-independent methods which present a severe limitation when studying antibiotic resistance.

In summary, the prevalence of antibiotic-resistant bacteria was different between the SML and UW only when selection was conducted using antibiotic-supplemented agar. Even so these results suggest that SML conditions select for antibiotic resistance. The overall results also showed that, although antibiotic resistance occurs among estuarine bacteria, the resistance mechanisms are different from the most common acquired mechanisms, being probably predominantly intrinsic. Although we cannot exclude the

contribution of transferable genetic elements, our results suggest that SML primarily selects for intrinsic antibiotic-resistant.

6. General discussion

Bacterioneuston communities are poorly characterized especially in what concerns the spatial and temporal factors that might influence its phylogenetic diversity and the metabolic activities they exert (Stolle et al., 2010).

Furthermore, studies comparing the bacterioneuston and bacterioplankton communities lack detailed information about the extent of diversity inside specific phylogenetic lineages that might highlight distinctive features of each community and at the same time, reveal to what extent the two compartments communicate, and what phenotypes and genotypes are shared.

In general, comparative analyzes on the bacterioneuston and bacterioplankton composition is often based on the phylogenetic diversity of the total community and evidences that have been gathered so far delivered contradictory findings (Agogué et al., 2005a; Franklin et al., 2005).

The work presented in this thesis has definitely contributed to extend our knowledge on the structure and dynamics of bacterioneuston communities from an estuarine environment, comparing to the underlying bacterioplankton and establishing some relations with the local environmental characteristics.

It is our conviction that this study filled some of the knowledge gaps in this field. For example, as the properties of SML may vary significantly along time and space (Peltzer et al., 1992; Santos et al., 2009), the temporal and spatial dynamics of bacterioneuston communities and specifically the temporal and spatial variation in terms of differences between bacterioneuston and bacterioplankton were evaluated. We followed changes on the structure and dynamics of the bacterioneuston and bacterioplankton communities in three geographical locations along *Ria de Aveiro* estuary (with different anthropogenic inputs) in four sampling dates, including spring, summer and autumn periods. Moreover, the first task of this research plan consisted on preliminary studies that allowed us to carefully assess to what extent spatial variability was observed, thus ensuring that the samples collected at each location were representative of each sampling site. For that, at each sampling site, samples were taken every 50 meters along a 200 meters transect. Furthermore, sampling was conducted at low and high tide and also during day (maximum light) and night (minimum light). Preliminary results based on fingerprinting analysis (DGGE) indicated that samples preferably grouped according to the sampling sites and that the bacterioplankton and bacterioneuston communities shared a high degree of homology

(data not shown). On the other hand the light regimens had a considerable influence in the structure of bacterioneuston. Regarding tidal regimens, all samples collected at low or high tides clustered separately. Thus, tidal regimens ought to be taken into account particularly in studies conducted in an estuarine environment, which is the case of the present study.

Estuarine systems are strongly influenced by anthropogenic activities accumulating high levels of pollutants and often becoming eutrophicated (Keddy, 2000). A variety of compounds often accumulate in the SML compartment, thus gathering ideal conditions for the establishment of a distinct estuarine bacterioneuston community. However, estuarine systems are very dynamic and the accumulation of organic matter including pollutants can be strongly altered by spatial and temporal changes. Thus, at low tide, the lowest volume of water in the estuary would facilitate the concentration of organic matter, consequently influencing the establishment of a distinct estuarine bacterioneuston, making this moment the ideal for SML sampling.

To the best of our knowledge, there are no other studies in this field that have considered these many aspects: the spatial and temporal dynamics, the tidal regimen, and the light regimen. Moreover, meteorological aspects such as wind speed and rainfall can strongly impact the formation of the SML and thus influence the obtained results when studying the bacterioneuston (Wurl et al., 2011). Hence, we were very cautious to avoid the influence of meteorological conditions: samples were always taken under a low wind speed (<4 m s^{-1}) and mild weather (without rainfall). Furthermore, in each campaign we ensured that samples were taken from all sampling sites within 24 to48 hours to avoid the influence of temporal aspects.

SML sampling devices must be carefully chosen and, if possible, more than one type should be used to minimise bias associated to each method (Agogué et al., 2004). In this specific study, given the high number of samples analysed, it was decided not to add samples obtained with another sampling device, thus avoiding increasing number of samples to non-manageable levels. After a revision of the available literature, we have chosen the sampling device that seemed to be the most suitable for our working conditions: the glass plate. As all SML samples were collected using always the same device, and this, in our opinion, represents an advantage in the sense that avoids biases introduced by sampling thus allowing some confidence in comparing results from different samples.

Furthermore, all samples were evaluated not only in terms of the total bacterial community (**Chapter 3**), but also focusing on the phylogenetic diversity of specific groups such as *Pseudomonas*, *Aeromonas* (**Chapter 3)** and *Psychrobacter* **(Chapter 4**) and functional diversity in terms of antibiotic-resistant bacteria in the SML and UW in *Ria de Aveiro* (**Chapter 5**). Again, to our knowledge this presents the broadest study ever conducted to analyse the bacterioneuston within the same aquatic system.

Additionally, the same aquatic system has been analysed by other authors that performed comparative studies on specific features that were distinct between the bacterioneuston and bacterioplankton. The main findings of those studies are reported on the next lines:

a) It has been hypothesized that, due to enrichment in organic matter and presence of a wide array of pollutants, higher rates of horizontal gene transfer could occur at the SML. To investigate this hypothesis Oliveira and coworkers analysed the presence and diversity of mobile genetic elements within the bacterioneuston and bacterioplankton communities from *Ria de Aveiro* (Oliveira et al., 2012). The authors captured novel plasmids conferring resistance to tetracycline and mercury, many of which were captured from the SML.

b) Another critical aspect that may contribute to the establishment of a different bacterial community in the SML is the fact that this environment is subjected to stronger solar radiation. The resistance to ultra-violet radiation of SML and UW communities has been thoroughly evaluated in *Ria de Aveiro* (Santos et al., 2011a,b; 2012).

c) Finally the accumulation of pollutants in the SML may have also selected for phylogenetic groups able to degrade and consume these compounds. This was assessed by Coelho and coworkers in what concerns the capacity to degrade aromatic compounds (Coelho et al., 2010, 2011).

The high amount of data collected and the inferences made from them, as well as the several aspects and issues considered in the studies cited above, make the aquatic system *Ria de Aveiro* as one of the most (if not the most) well studied environments in what concerns the characterization of the bacterioneuston and the comparison between bacterioneuston and bacterioplankton. Also the phylogeny of the bacterioplankton communities within *Ria de Aveiro* had previously been extensively characterised by using

culture-dependent and culture-independent approaches (Henriques et al., 2004; 2006a,b,c; 2008). In summary, those studies have revealed that phylogenetic shifts within the bacterioplankton community occurred essentially between the brackish and freshwater sections in the estuary. Furthermore, seasonally driven changes in such bacterioplankton community were also registered. Moreover, antibiotic resistance within *Ria de Aveiro* bacterioplankton had been also investigated and results indicated the occurrence of a diversity of resistance mechanisms and molecular variants of genes conferring antibiotic resistance, which potentially classified the estuary as a natural reservoir of antibiotic resistance genes (Henriques et al., 2006b,c).

Most molecular methods of microbiological analysis used in this thesis project have been previously validated by our research group on samples from the same estuary during the characterization of bacterioplankton community (Henriques et al., 2004; 2006a,b,c; 2008). The combination of culture-dependent and culture-independent methodologies was considered successful in such studies in the sense that contributed to overcome the drawbacks commonly associated to each approach. Those methodologies have shown to be reliable for the simultaneous analysis of large numbers of samples and to monitor changes in bacterial community structure according to spatial and temporal factors.

A strong reason for using both culture-dependent and culture-independent methods in this study is that most studies that have examined differences between SML and UW communities in the last decade were driven by the idea that molecular approaches are able to give a complete picture of the microbial composition in any environment and consequently, were limited to culture-independent approaches (Agogué et al., 2005a; Franklin et al., 2005; Cunliffe et al., 2009a).

Culture-independent approaches have the advantage to provide a better picture of total bacterial community structure. In fact, *16S* rDNA DGGE profiles revealed that differences between both communities remained relatively constant as previously reported (Cunliffe et al., 2008). Even so, our results showed that differences between both communities frequently vary according to spatial and temporal factors. On the other hand, the culture-based approach led to the successful cultivation of a considerable diversity of heterotrophic bacteria. This community is expected to be of major importance in the SML due to the high amounts of organic matter ought to occur. By using a culture-dependent approach differences (although minor) between both communities were again highlighted.

The combination of culture-dependent and culture-independent methods was also very effective on the description of *Psychrobacter* phylogenetic diversity. A genus-specific-DGGE-based method (designed during this study) and genus-specific cloning libraries for *Psychrobacter* were efficient in revealing that *Psychrobacter* populations are complex (high diversity) and very stable in all sites and sampling dates. This stability might indicate the presence of well-adapted phylotypes in this aquatic environment. The culture-dependent approach contributed to the characterization of this diversity and also provided a *Psychrobacter* culture collection in which presumably new species are included. The characterization of these new taxa was already started and will soon be published. A possible drawback of this study was the lack of quantitative data on the abundance of *Psychrobacter* along the estuary. In future studies it is advisable to use for example quantitative PCR specific for *Psychrobacter* to assess the abundance of this genus in *Ria de Aveiro* and to correlate this abundance with environmental variables that might be contributing for the successful colonization of this temperate habitat by this cold-adapted genus.

In terms of functional diversity, we intended to evaluate the contribution of the bacterioneuston community to the antibiotic resistance gene pool and antibiotic resistance dissemination. We hypothesize that under the conditions occurring at the SML (enrichment of pollutants and organic matter, the formation of a gelatinous film that probably supports a biofilm) horizontal gene transfer and the dissemination of antibiotic resistance is more plausible to occur than in underlying waters.

We found a very low incidence of known antibiotic-resistance genes, when compared to what was previously reported for bacterioplankton communities (Henriques et al., 2006c; 2008). We are aware that this "low incidence" might be associated to the culture-dependent criteria applied in this work, which was non-antibiotic selective.

On the other hand, this study was able to characterize the antibiotic resistance profile among a high diversity of phylogenetic groups present in the heterotrophic bacterial communities in *Ria de Aveiro*. This result opens new possibilities to characterise the mechanisms and genetic elements involved in antibiotic resistance in SML and UW communities.

Moreover, further studies concerning antibiotic resistance should compare the particle-attached and non-particle-attached bacterioneuston with bacterioplankton community.

According to Wilson et al. (2010) and Hakvåg et al. (2008) antagonistic activity have often been described among particle-attached bacteria, thus this aspect could highlight differences between both water layers.

Question on whether bacterioneuston constitutes a distinct community or is closely related to bacterioplankton is far from being completely answered. However, our results definitely provide additional and consistent information regarding both microbial communities' compositional structure and dynamics by combining culture-dependent and independent surveys.

Summary of conclusions

This study definitively contributed to clarify the similarity/divergence between the communities of bacterioneuston and bacterioplankton in *Ria de Aveiro*. Most of results and conclusions can be easily extrapolated to other aquatic systems, adding knowledge to a field that has been poorly explored. Specifically differences between SML and UW communities probably occur in most (if not all) the aquatic systems, although in some cases these differences are not pronounced. The fact that spatial and temporal factors influence comparative analysis between both bacterial communities is probably a global aspect, independently of the aquatic system being considered. Also, in terms of antibiotic resistance, the higher prevalence of antibiotic-resistant bacteria in the SML may also be a common feature, owing to the selective conditions in this layer. On the other hand, the specific phylogenetic groups enriched in the SML or the UW may be dependent on the specific conditions of each ecosystem. In fact environmental conditions such as salinity or temperature and anthropogenic pressures such as the presence of specific contaminants obviously determine the composition of SML and UW and the differences between these communities.

Results allowed stating the following conclusions:

Spatial and temporal analysis of estuarine bacterioneuston.

✓ DGGE profiles indicated that SML and UW communities are highly similar; however SML-specific phylotypes were identified.

✓ Most of the SML exclusive DGGE-bands were detected during day on the same sampling site, suggesting that differences between SML and UW communities are probably related to spatial and diel factors.

✓ Structural and compositional differences between SML and UW aeromonads communities were not identified.

✓ Compositional differences between both bacterial communities based on culture-dependent survey were confined to a few genera overrepresented in UW samples (*Kocuria*, *Agrococcus* and *Vibrio*).

✓ A cultivable operational taxonomic unit affiliated to *Pseudomonas* was consistently overrepresented in SML samples.

Psychrobacter* populations within *Ria de Aveiro

✓ The culture-dependent and culture-independent approaches were an asset to the description of the diversity of genus *Psychrobacter*

✓ A collection of cultivable *Psychrobacter* isolates was obtained containing strains representing putative new species. The characterization of novel species is currently on-going.

✓ A surprisingly high diversity among *Psychrobacter* in *Ria de Aveiro* was found suggesting that this genus is well-adapted to this environment.

Antibiotics resistance in two water layers

✓ A very low incidence of known antibiotic-resistance genes was found that might be associated to the culture-dependent criteria, which was non-antibiotic selective.

✓ Higher prevalence of antibiotic-resistant bacteria was found in the SML suggesting that SML conditions select for antibiotic resistance.

✓ Antibiotic resistance was uncommon among estuarine bacteria and the resistance mechanisms were probably predominantly intrinsic.

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