Universidade de Aveiro Departamento de Biologia
2009

Isabel Cristina Santos Silva de Faria Ramos

Comunidade bacteriana cultivável da microcamada superficial estuarina

Culturable bacterial community of the estuarine surface microlayer

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Prof. Dra. Isabel Henriques, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho à minha família por todo o apoio e compreensão.

o júri

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palavras-chave

Bacterioneuston, bacterioplâncton, métodos dependentes do cultivo, 16S rDNA, ARDRA, diversidade filogenética.

resumo

A camada superficial aquática (1-1000 µm) é um ecossistema único, definido como a interface entre a hidrosfera e a atmosfera. É uma camada exposta a altas intensidades de radiação solar Ultra-Violeta, sendo enriquecida com compostos orgânicos e poluentes antropogénicos. Além disso, está sujeita a condições instáveis de temperatura e salinidade.

Assim sendo, é razoável colocar-se a hipótese de que esta camada é habitada por comunidades bacterianas distintas e especializadas. Apenas alguns estudos sobre este tema foram publicados e os resultados foram frequentemente divergentes. Apesar do já reconhecido enviesamento introduzido pelas metodologias dependentes do cultivo, tais técnicas permanecem essenciais para a compreensão da fisiologia e ecologia da comunidade bacteriana.

Os estuários são ambientes confinados e frequentemente muito poluídos, o que provavelmente favorece a formação de camadas superficiais claramente distintas das águas subjacentes. Portanto, o objectivo deste trabalho foi comparar as comunidades bacterianas cultiváveis da camada superficial aquática e da coluna de água. Foram escolhidos três locais ao longo do estuário Ria de Aveiro atendendo a diferentes parâmetros ambientais e exposição a poluentes. A amostragem foi realizada utilizando o método 'Glass-Plate'. As amostras foram obtidas em maré baixa, durante o dia e noite, em cinco campanhas, tendo em vista a quantificação das unidades formadoras de colónias e subsequente isolamento para caracterização filogenética. Para estes fins, usámos dois meios de cultura: GSP (*Pseudomonas Aeromonas* Selective Agar Base) e EA (Estuarine Agar).

A quantificação das UFC indica que o número de bactérias provenientes da camada superficial (bacterioneuston) é cerca de três vezes mais abundante do que o proveniente da coluna de água (bacterioplâncton). Verifica-se uma diminuição da abundância de bacterioneuston de dia para noite, ao contrário do bacterioplâncton, que tende a aumentar durante o mesmo período. Dos isolados obtidos, o rDNA 16S foi e digerido com a enzima *Hae*III. A partir de 402 isolados, foram identificados 72 perfis diferentes. Desses, 21 perfis foram exclusivos da camada superficial e 28 foram exclusivos da coluna de água. Representantes dos diferentes perfis foram analisados por sequenciação e bactérias pertencentes a 5 Filos: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria, Firmicutes* e *Deinococci-Thermus*; e 9 Classes: *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Actinobacteria, Flavobacteria, Sphingobacteria, Deinococci* e *Bacilli* foram identificadas. Os isolados afiliaram com sequências provenientes de ambientes aquáticos bem como de áreas altamente contaminadas. Os resultados apontam para uma comunidade cultivável distinta/particular na microcamada superficial estuarina.

keywords

Bacterioneuston, Bacterioplankton, culturable-dependent methods, 16S rDNA, ARDRA, phylogenetic diversity.

abstract

The sea surface microlayer (SML) is an unique ecosystem, defined as the interfacial film (uppermost 1–1000 μm) between the atmosphere and the ocean. Thereby, it is exposed to high intensities of solar radiation, and is enriched with organic compounds and pollutants from anthropogenic inputs. Also it is subjected to unstable temperature and salinity conditions.
Thus, it is proper to hypothesize that the SML is inhabited by distinct and specialized microbial communities. Only a few studies on this topic were published and results were frequently divergent. Despite the previously recognized biases introduced by culture-dependent methodologies, such techniques remain essential to understand bacterial population's physiology and ecology.
Estuaries are confined and frequently highly polluted environments, which probably favor the formation of distinct surface layers clearly distinct from underlying waters. Therefore, our goal was to compare the culturable bacterial communities occurring in SML and underlying waters (UW). Our work concerned three sampling sites in the estuary Ria de Aveiro, corresponding to different environmental parameters and exposure to pollutants. Sampling was conducted using the so-called 'Glass-Plate' method. The UW samples were collected directly into a sterilized glass bottle from a depth of approximately 0.4 m. Samples were obtained at low-tide, during day and night, in five campaigns, regarding the CFU (Colony Forming Units) quantification and subsequent recovery of bacterial isolates. For these purposes we used two culture media: GSP (*Pseudomonas Aeromonas* Selective Agar Base) and EA (Estuarine Agar).
CFU quantification indicates that bacterioneuston is about three times more abundant than bacterioplankton. Generally bacterioneuston abundance decreases from day to night while bacterioplankton usually increases during the same period. From all the obtained isolates the 16S rDNA was amplified using universal primers and digested with the enzyme *Hae*III. The profiles were analyzed using the software GelCompar and representatives of each pattern were selected for sequencing. From 402 isolates, 72 different profiles were identified. From those 21 profiles were exclusive from SML samples and 28 were exclusive from UW samples. Sequencing results allowed identifying bacteria belonging to 5 different Phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria, Firmicutes* and *Deinococci-Thermus*; and 9 Classes: *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Actinobacteria, Flavobacteria, Sphingobacteria, Deinococci* e *Bacilli*. Isolates affiliated with sequences from aquatic environments as well as highly contaminated areas. The results point to a distinct/particular culturable community within the SML of this estuarine environment.

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LIST OF ACRONYMS AND ABBREVIATIONS

µg	microgramms
µl	microliter
µm	micrometer
BN	Bacterioneuston
bp	base pair
BP	Bacterioplankton
CC	Cais do Chegado
CFU	Colony Forming Units
CH_4	Methane
CN	Costa Nova
CO	Carbon monoxide
CO_2	Carbon dioxide
CS	Cais do Sporting
DAPI	4,6 – diamidino – 2 – phenylindole
DMS	Dimethilsulphide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosides
DOC	Dissolved organic carbon
EF	Enrichment Factors
ε-proteobacteria	*Epsilonproteobacteria*

GP	Glass-Plate
Km	Kilometer
L	liter
m	meter
mg	miligramm
$MgCl_2$	Magnesium Chloride
min	minute
ml	mililiter
mM	miliMolar
mm	milimeter
MS	Metal Screen
N_2O	Nitrous oxide
NaCl	Sodium Chloride
ng	nanogramm
nm	nanometer
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
pmol	picomole
POPs	Persistent Organic Pollutants
RD	Rotating Drum

RNA	Ribonucleic acid
SML	Sea Surface Microlayer
TAE	Tris – Acetate - EDTA
TBT	Tributyltin
TE	Tris - EDTA
TPT	Triphenyltin
UW	Underlying Waters
α-proteobacteria	*Alphaproteobacteria*
β-proteobacteria	*Betaproteobacteria*
γ-proteobacteria	*Gammaproteobacteria*

Introduction

1. Introduction

Former works designed to study the sea surface microlayer (SML) have focused mainly on its chemical composition (Carlson, 1982; Hardy *et al*., 1985; Maki, 1993), while spatial and temporal characteristics of SML biological communities have received slight attention. Therefore, in the past five years, a few studies on the SML biological communities have emerged, but the results were often divergent (Agogué *et al.*, 2005; Franklin *et al*., 2005; Cunliffe *et al*., 2008; Hervas and Casamayor, 2009). In addition, a limited number of studies have focused on the diversity or structure of SML bacterial culturable communities (Agogué *et al*., 2005; Joux *et al*., 2006).

1.1. The sea surface microlayer: physicochemical characterization

Aquatic surface microlayers exist ubiquitously in both marine and freshwater environments (Hale and Mitchell, 1997). The SML is a thin biogenic film that can be found in the surface of the ocean, having an estimated depth of 1000 μm (Liss and Duce, 1997). However, it is usually defined by the depth sampled, which depends on the applied sampling device (Agogué *et al*., 2004). This layer is physically, chemically and biologically distinct from underlying waters (UW), which have been defined as the water layer at depth of >1000 μm (Lion and Leckie, 1981; Zhang *et al*., 1998, 2003; Zhengbin *et al*., 1998).

As most important chemical processes occur at surfaces or interfaces between different environments, SML has a very important role (Hardy, 1982), adding the fact that it makes up 70% of the total earth surface area and provides the physical link between the sea surface and the lower atmosphere (Liss and Duce, 1997). In fact, a boundary layer at the water-atmosphere interface was reported to play a role of main authority on exchanges of matter and energy at several temporal and space scales (Liss and Duce, 1997).

An understanding of the processes and physical mechanisms governing the exchange of gases between air and sea is essential when considering coupled models of atmosphere-ocean interactions and global climate, and these interactions are of particular concern as they may affect or be affected by global change. Indeed accurate estimates of air-sea gas fluxes are essential for understanding the global cycles of carbon dioxide (CO_2), methane

(CH_4), nitrous oxide (N_2O), dimethylsulphide (DMS) and other trace gases that affect the earth's radiation budget. For instance, the sea carbon inventory is about fifty times larger than the amount of atmospheric CO_2 – as we know a critical greenhouse gas. Also, glaciated areas studies reported larger variations in atmospheric CO_2 (~25%) over glacial-interglacial cycles, being the ocean the most probable perpetrator for these oscillatory events (Sigman and Boyle, 2000). Sarmiento and Gruber enhance the fact that about a third of the CO_2 released by fossil fuel combustion is absorbed by the ocean, highlighting the potential greenhouse warming (Sarmiento and Gruber, 2002).

We cannot forget that there are also significant biological aquatic sources of N_2O – also a greenhouse gas and a key compound in stratospheric ozone chemistry – CH_4 and DMS, which can alter cloud and radiation feedbacks (Charlson *et al*., 1987).

The intricate dynamics of biological responses to environmental pressures and interspecies interactions on the aquatic environment has not yet been totally discovered and this raises upsetting issues on our capacity to forecast the future behavior of such a system under climate warming and to prevent irreversible damages (Doney *et al*., 2004).

Most of the organic matter in water is synthesized by photosynthetic microbes while in the other hand heterotrophic microbes degrade it. These microbial activities regulate the ocean biogeochemical cycling, namely redox state, nutrient cycling and trace gases relevant to global climate: CO_2, DMS, N_2O. Indeed, aquatic biology concerning its biogeochemistry and climate are poorly understood but it represents an important constituent of the global climate system (Doney *et al*., 2004).

Hunter, in 1980, had described SML as an upper hydrodynamic boundary layer with a thickness of ~50 µm (Hunter, 1980). Six years later, Hardy and Word (1986) defined three distinct layers within the SML (figure 1):

- the surface nanolayer (<1 µm), the most superficial, where surface-active substances were found;
- the surface microlayer (<10 µm), containing enriched particles and being inhabited by microorganisms;
- the surface millilayer (<1000 µm),the habitat for fish eggs and larvae (Hardy and Word, 1986).

More recently, according to Zuev *et al*. (2001), the SML has the following structure (from top to bottom): a monomolecular lipid film (10-20 μm), a polysaccharide-protein layer (100-300 μm), a layer of suspended abiotic particles, and bacterioneuston and deeper layers of phytoneuston and zooneuston (Zuev *et al*., 2001).

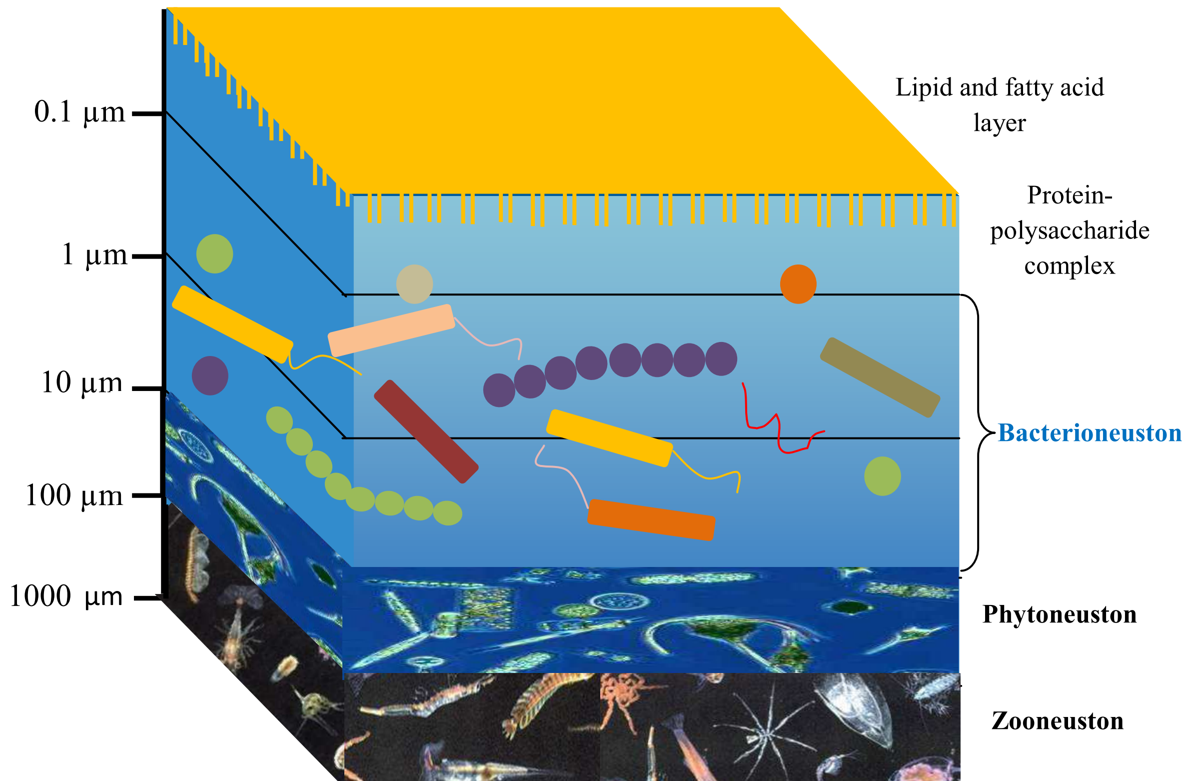

Figure 1: Schematic representation of SML stratification attending to Hardy and Word's model (Hardy and Word, 1986).

In the final of the last century, some of the authors that more contributed to unravel this topic were Zhang and colleagues, describing a multiple-layer model, within which physicochemical properties of the SML modify swiftly. The thickness of SML in the multiple-layer model was empirically determined to be 50 ±10 μm (Zhang *et al*, 1998).

A few years later, Zhang *et al*. (2003) established the thickness of the SML as roughly 60 μm, based on measurements using pH-microelectrodes; however, the authors did not

exclude the possibility of SML's thickness variations due to modifications in meteorological conditions and organic matter enrichment on this layer (Zhang *et al*, 2003). In summary, the thickness of the SML remains a topic of discussion.

The SML also differs from UW in terms of temperature. The ocean and atmosphere are not in thermal equilibrium, with the surface layer of the ocean (top 100 µm) being on average 0.1-1.4ºC warmer than the troposphere, owing to continuous solar radiation and the different optical characteristics of these environments (Zuev *et al.*, 2001).

It is well established that the SML is a unique environment with substantial unpredictability of chemical characteristics, when compared with the UW (Reinthaler *et al.*, 2008). As previously stated, the air-water interface is a place of energetic exchange due to processes of gaseous, liquid and particulate matter transfer between the atmosphere and aquatic environments (Liss *et al.*, 1997). On a recent study by Walczak and Donderski (2004) the SML was reported to be a complex layer with a mixture of lipids, proteins, sugars and their derivates as well as other substances that concentrate at this boundary (Walczak and Donderski, 2004). In fact, there are numerous compounds that preferably deposit in the SML such as nutrients, dissolved organic carbon (DOC), and amino acids (Williams *et al.*, 1986). In fact, it had early been reported that enrichments in organic matter at SML could be 1000 times higher when compared to the UW (Hardy, 1982; Liss and Duce, 1997).

SML's enrichments have been endorsed to a series of physical and biological processes including diffusion, convention, turbulent mixture and *in situ* primary productivity (Walczak and Donderski, 2004). Kuznetsova and Lee pointed out another important process that contributes to these enrichments: rising gas bubbles (Kuznetsova and Lee, 2002). Aquatic aerosols originate from bubbles and can be easily suspended and transported in the lower atmosphere (Woodcock, 1953; Gustafsson and Franzen, 2000; Grammatika and Zimmerman, 2001). Major sources of bubbles are waves and rain impacting the sea surface (Monahan *et al.*, 1983; Klassen and Roberge, 1999). Therefore, bubbles can be dispersed to depths of several meters (Grammatika and Zimmerman, 2001) transporting dissolved surface-reactive inorganic and organic compounds, metals and small particles including bacteria and viruses adherent to its surface. Also, these bubbles ultimately ascend through the water column and reach the sea surface, disintegrate and expel aerosol droplets into the atmosphere, delivering the material carried by the bubbles to

the surface microlayer and to the atmosphere (Blanchard, 1975; Blanchard and Syzdek, 1982).

More recently, atmospheric deposition of matter has also been reported to be a very important input for the enrichment of SML (Wotton and Preston, 2005).

One of the consequences of the presence of high amounts of organic compounds in the SML is a decrease in surface tension (Zuev *et al*., 2001) and, on the other hand, an increase in film stability, which promotes an enrichment in particles, organisms and dissolved material. In fact, the SML's chemical composition defines its elasticity: lower productivity waters show a decrease in the protein/lipid ratio in the SML, subsequently increasing its elasticity (Frew and Nelson, 1992).

SML's chemical composition is also characterized by a thick spectrum of diverse compounds, which may not be soluble in water and therefore be extractable with organic solvents. In fact, one can count as many as 16 different compounds being brought by natural or anthropogenic inputs: aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, wax esters, sterol esters, short-chain esters, acylated glyceryl ethers, triglycerides, free fatty acids, phthalate esters, free aliphatic alcohols, sterols, diglycerides, monoglycerides, glycolipids, pigments and phospholipids (Parrish *et al*., 2000).

Chemical contaminants are known to accumulate in the SML due to its unique physicochemical properties (Liss, 1975; Hardy, 1982). In the literature we found several studies on the following SML pollutants that preferably accumulate on SML: pesticides, polychlorinated biphenyls (PCBs), organotin compounds, petroleum hydrocarbons (like Polycyclic Aromatic Hydrocarbons (PAHs)) and heavy metals (El Nerm, 2003; Mudryk, 2004; Wurl and Obbard, 2004; García-Flor *et al*., 2005; Guitart *et al*., 2007; Lim *et al*., 2007; Cuong *et al*., 2008).

Persistent organic pollutants (POPs), such as pesticides and PCBs, are xenobiotic chemicals and represent a threat since these compounds are recalcitrant and potentially toxic. Therefore, the dispersion of POPs and their impact on a wide range of ecosystems has been an issue of concern (Jeminez, 1997; Jones and de Voogt, 1999). In fact, POPs have already been implicated in causing undesirable effects on endocrine systems in an extensive sort of aquatic organisms, including mammals (Tanabe, 2002; Bosveld and van den Berg, 2002).

Another potentially toxic pollutant is organotin, namely, tributyltin (TBT) and triphenyltin (TPT) that have been extensively used as antifouling boat paints since the early 1970s and it is kwon that preferably accumulate within the SML (Gucinski, 1986). As a result, large quantities have been introduced in the aquatic environment (Berg *et al*., 2001; Hoch, 2001). Both TBT and TPT were reported to be harmful to aquatic life (Hoch, 2001). For instance, even at low nanomolar aqueous concentrations (1–2 ngl^{-1}), TBT causes chronic and severe toxic effects in most susceptible aquatic organisms, such as algae, zooplankton, molluscs and the larval stage of some fishes (Gibbs and Bryan,1996).

The contamination of aquatic environments by PAHs is also a matter of concern since they are among the most carcinogenic, mutagenic and teratogenic contaminants (Kennish, 1992). Generally these compounds are found at higher concentrations in the SML, frequently being associated with anthropogenic coastal activities, particularly shipping harbours (Cross *et al*., 1987; Hardy *et al*., 1990; Kucklick and Bidleman, 1994; Zeng and Vista, 1997; Cincinelli *et al*., 2001). For example, a recent study reported levels of PAHs contamination at the SML five times higher than at UW (El Nemr, 2003).

Heavy metals comprise a group of elements crucial to the metabolism of many aquatic organisms but they are potentially toxic above threshold bio-available levels (Blackmore 1998). The input of heavy metals in aquatic environments has more than a few sources including atmospheric fallout (consisting of both wet and dry deposition), riverine inputs, wastewater discharges and re-suspension of contaminated sediments (Mart *et al*., 1982; Poikane *et al*., 2005). The magnitude of heavy metals concentrations in polluted areas at SML's can be about 1000 times higher, when compared to those measured in UW samples (Cuong *et al*., 2008).

In terms of toxicity, one cannot forget that SML provides the habitat for marine biota, including fish eggs and the larvae of many commercially important fish species. The accumulation of organic pollutants and heavy metals in the SML leads to ecotoxicological impacts to the upper layer community including mortality, developmental abnormalities, depression of growth rates and prolonged hatch time of fish eggs (Wurl and Obbard, 2004). Obviously these levels of contamination also impact the microbial communities present in the SML, namely the bacterial communities.

Overall, it is obvious that the quality of the SML has been ruined in the human highly impacted coastal regions (Wurl and Obbard, 2004).

1.1.1. Biological characteristics

Although the SML represents an extreme environment for living organisms, it is the habitat for a wide spectrum of organisms ranging from virus to plants or fish and is generally enriched in bacteria, microalgae, yeasts, molds, and protists (Hardy, 1982; Williams *et al*., 1986).

Early on the twenty century, Naumann named the collection of organisms inhabiting the SML as neuston; the bacterial community was called bacterioneuston (BN) (Naumann, 1917). These organisms are supported by the abundance of organic matter that can be found at the sea surface. Organisms from most major divisions of the plant and animal kingdoms live, reproduce or feed in the SML. Permanent inhabitants of the SML include bacterioneuston, phytoneuston and zooneuston, such as small metazoan and large metazoan and eggs, larvae and small fishes (Zaitsev, 1971).

In the past ten years, several reports have revealed that SML marine aerosols contain viruses (e.g. Klassen and Roberge, 1999; Moorthy *et al*., 1998; Chow *et al*., 2000).

Phytoneuston is part of the ocean autotrophic community, wich is responsible for the base of the aquatic food web, playing an essential ecological function (Montes-Hugo and Alvarez-Borrego, 2007). The species composition of microalgae in the SML frequently differed from that in UW (Williams *et al*., 1986; Hardy & Apts, 1984). The tendency towards a dominance of small flagellates and small pennate diatoms in the SML has been previously reported (Hardy, 1973).

Accordingly, zooneuston (copepods and fish larvae for example) has a critical role at the food aquatic network being consumed by higher trophic levels and also responsible for the consumption of phytoneuston, controlling, for instance, the seasonal blooms of the latter (Hardy & Apts, 1984).

Karner and colleagues have estimated that uncultured *Archaea* in marine ecosystems ranged from 30% to 40% and in freshwaters from 1 to 20% (Pernthaler *et al*., 1998; Glockner *et al*., 1999; Keough *et al*., 2003; Urbach *et al*., 2007). Recently, Auguet and colleagues, in a study concerning high mountain lakes reported 4,6-diamidino-2-phenylindole (DAPI) counts for *Archaea* inhabiting SML ranging between 3% and 37%. Community was dominated by *Crenarchaeota* of a new freshwater cluster distantly related to the Marine Group 1.1a. (Auguet and Casamayor, 2008)

The SML's biological activity is far from being understood. For instance, the potential contribute to air-water exchange processes has been studied and the partial pressure of CO_2 in the upper film of the ocean was reported to be correlated to the microbial community metabolism (Calleja *et al*., 2005). In addition, this theory is supported by the increased rates of respiration in samples collected from SML at different coastal and near shore aquatic environments (Garabétian, 1990; Obernosterer *et al*., 2005). Reinthaler and colleagues found, more recently, higher bacterial respiration rates in the SML, supporting the idea of biological control of CO_2 fluxes across the SML (Reinthaler *et al*., 2008).

Indeed, the SML influence in climate and atmospheric chemistry of Earth is highly relevant and that is why the study of the active involvement of the SML bacterial community in the air–sea exchange of CH_4 and CO is so important (Cunliffe *et al*., 2008).

These biological processes are prone to depend on the spatial and temporal variability in the development of the SML (Obernosterer *et al*., 2007).

1.1.2. Bacterioneuston: the bacterial community inhabiting the SML

Despite the long-lasting interest in the physicochemical properties of the SML, studies on the bacterial community structure and activity are still scarce. For bacteria, the SML might be a stressful environment. The SML has, as said before, preferential deposition of organic matter and pollutants in comparison with the UW. On the other hand, it receives intense solar irradiation, especially in the low wavelength range of ultraviolet-B (300–320 nm), which is generally disadvantageous to organisms (Regan *et al*., 1992; Agogué *et al*, 2005).

Nevertheless, several studies reported the abundance of the bacterial population to be 10^2-10^4 times higher at SML when compared to UW (Harvey, 1966; Morita and Burton, 1970; Sieburth, 1971; Tsyban, 1971; Sieburth *et al*., 1976; Münster *et al*., 1998).

Bacterioneuston structure and phylogeny is poorly understood. In fact, only a few reports are available in this area; six works were recently published on an attempt to clarify this issue (Agogué *et al*, 2005; Franklin *et al*., 2005, Joux *et al*., 2006; Cunliffe *et al*., 2008; Obernosterer *et al*., 2008; Hervas and Casamayor, 2009).

Franklin and colleagues (2005) performed a survey on the coast of Northumbria in the North Sea intending to compare the phylogenetic diversity and structure of bacterial community from SML to those from the UW. SML was sampled with 47 mm diameter and 2 μm pore polycarbonate membranes to avoid possible contamination of the samples that could interfere with the sensitivity of Polymerase chain reaction (PCR). So, environmental DNA was extracted from three campaigns and culture-independent methods were applied. Then, 16S libraries of about 500 clones from both SML and UW were analyzed. SML samples displayed lower bacterial diversity when compared with those from UW samples. Results also showed that SML comprises a distinct population that differs considerably from the bacterial population of UW. Therefore, the authors stated that, according to molecular taxonomic evidences, BN is a distinct bacterial community (Franklin *et al*., 2005).

During the same year, Agogué *et al*. performed a research where samples were obtained from two coastal sites in the north-western Mediterranean Sea for a two years period, and culture-dependent techniques were applied. In this study several types of samplers were applied to SML sampling but no significant differences were found within the obtained results. 487 isolates were recovered, analyzed by genetic fingerprinting and identified by sequencing the 16S rRNA gene. The sequencing analysis showed no reliable differences between BN and Bacterioplankton – de bacterial community inhabiting UW (BP). Remarkably new marine genera were brought into culture (Agogué *et al*., 2005).

Joux and colleagues performed a study in 2006 which aimed to compare the microbial community structure between SML and UW, determining the enrichment factors (EF) of 13 biological parameters. Samples were taken at 2 contrasting coastal sites in the Mediterranean Sea, corresponding to a high (Barcelona, Spain) and low (Banyuls-sur-Mer, France) urbanized area. Sampling was performed with the metal screen. Attending to the culturable fraction of bacteria, this study found that abundance and activity of the parameters determined in the SML was highly correlated with that determined in UW, supporting the idea that enrichment of the SML results mainly from upward transport of microorganisms attached to buoyant particles or bubble scavenging (Joux *et al*., 2006)

In 2008, a survey on the bacterial community inhabiting the SML and the UW from an estuarine environment (Blyth River at the North Sea) was conducted by Cunliffe and colleagues. Samples were obtained using the same samplers used in Franklin and

colleagues study (Franklin *et al*., 2005) from two sampling sites along a salinity gradient and culture-independent methods were applied. Denaturing Gradient Gel Electrophoresis was performed and obtained profiles from BN and BP shared about 88% similarity; therefore, the composition of BN and BP was found to be relatively similar. The 16S library results pointed for high BN diversity in disagreement with the first cited work by Franklin (Franklin *et al*., 2005; Cunliffe *et al*., 2008).

The biochemical composition and the bacterial community structure of SML from the South Pacific Ocean were determined in a study of Obernosterer and colleagues in 2008. SML was sampled at 6 stations ranging from oligotrophic to ultraoligotrophic using a metal screen. Culture-independent methods were applied and the obtained results from the fingerprints and the in situ hybridization suggest that the SML is not inhabited by a particular BN community (Obernosterer *et al*., 2008).

Finally, Hervas and Casamayor (2009) conducted a very singular study at a high mountain lake (Lake Redon, Pyrenees, Spain), raising an interesting hypothesis: are bacteria inhabiting the aquatic microlayer more related to airborn bacteria rather than to bacteria from UW? Again 16S libraries were constructed and analyzed. Results showed higher similarity between BN and the airborn community than between BN and BP (Hervas and Casamayor, 2009).

1.1.3. Sampling methods

The most suitable technique for SML sampling has been an issue of controversy over the past forty years. As we said before, due to the SML's dynamics and physical, chemical and biological heterogeneity, the collection of samples is one of the biggest challenges. For instance, the thickness of SML changes according to wind speed, which wave action possibly will perturb or even destroy the integrity of SML. Indeed, the employment of different sampling devices implicates thickness variations that probably contribute to the differences observed between different studies.

There are several sampling devices available, as listed in table 1:

Table 1: Sampling devices comparison adapted from Franklin *et al*., 2005

Sampling devices	Sample thickness (µm)	Sample collected	References
Freezing probe	1000	Sea water and particles	Hamilton and Clifton (1979)
Metal Screen (MS)	150-400	Microbes, lipids and fatty acids	Sieburth (1965); Garret (1967)
Rotating Drum (RD)	60-100	Microbes and organic matter	Harvey (1966)
Glass Plate (GP)	20-100	Chemical compounds and microbes	Harvey and Burzell (1972)
Hydrophilic Nucleopore membrane	4-40	Microbes and organic matter	Crow *et al.* (1975)
Hydrophobic Nucleopore membrane	20-50	Microbes and organic matter	Kjelleberg *et al.* (1979)
Bubbles microtome	1	Fractionated chemical and microbiological aerosol over sea surface	MacIntyre (1968)

The most commonly used sampling methods are Membranes, the Metal Screen (MS), the Glass-Plate (GP) and the Rotating Drum (RD) (Daumas *et al*., 1976).

Freezing probe technique consists in a probe with liquid nitrogen in which microlayer and particulate materials are detached by very rapid freezing to a depth of about 1000 µm, through a process that takes place in less than 1second (Hamilton and Clifton, 1979).

Metal screen consists in a stainless steel screen lowered vertically through the water surface, then oriented horizontally and lifted up through the SML (Garrett, 1965; Daumas *et al*., 1976).

Rotating drum sampler uses a smooth, gyratory cylinder whose surface is readily wet by water. A large neoprene blade is pressed tightly to 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).

Glass-Plate consists in an acrylic or glass plate that is vertically immersed into the water stream and then the adherent water (from the SML) is recovered to a sterilized bottle (Harvey and Burzell, 1972).

The hydrophilic membranes floated on the water surface, with adhering surface film, are retrieved by submerging sterile plastic dishes under them and delicately removing the filter and the UW; the membrane is then removed from the dish with forceps (Crow *et al.*, 1975). In contrast, Kjelleberg and colleagues used hydrophobic membranes, following basically the same procedure but its principal relays on the hydrophobic characteristics of bacteria for sampling (Kjelleberg *et al.*, 1979).

The bubbles microtome sampling is based on the fact that vertically expelled jet drops from bubbles smaller than 1 mm in diameter are formed from a thin superficial layer of liquid accelerated inward by surface forces. The drops are easily collected and offer a novel non-mechanical surface microtome (MacIntyre, 1968).

Two recent works were performed in order to compare the different specificities and bias from several types of samplers (Agogué *et al.*, 2004; Momzikoff *et al.*, 2004).

Both pointed out a real drawback for sampling with membranes is the selective adsorption of bacteria, which consequently overestimates the bacteria that are present in the SML; so, this kind of information should be carefully considered (Agogué *et al.*, 2004; Momzikoff *et al.*, 2004).

In the study conducted by Agogué and colleagues was demonstrated that the MS and the GP are proper for sampling both total and culturable bacteria and viruses and for bacterial activity and microbial communities' structure investigation (Agogué *et al.*, 2004).

Nowadays, MS and GP are the most frequently used devices. The comparison of studies that applied these two samplers implies looking to the results minding the difference in thickness of the sampled layer to avoid misinterpretation of the results (Agogué *et al.*, 2005; Joux *et al.*, 2006; Obernostere *et al.*, 2007).

On one hand, authors affirmed that GP is preferable when we want to collect the closest biological composition to the original in the SML. On the other hand, MS gives a level of contamination much lower than GP because has a smallest contact area between the water surface and the sampler (Agogué *et al.*, 2004).

One detail of great importance is the time spent in sampling, because the longer the time the higher the effect of temporal variability, which is relevant for example when

sampling systems subjected to tidal regimens. According to that, MS collects larger volumes in shorter periods of time (approximately 10 L per hour and per screen). In contrast, the GP has a much lower sampling capacity (1 L per hour and per plate) (Agogué *et al.*, 2004).

In summary, the most suitable sampling device depends on the aims of the work. The depth at which samples of UW are collected should be also cautiously controlled at each sampling because variations in the depth may implicate several shortcomings, such as differing enrichment factors.

1.2. Studying the bacterial communities: culturable vs. unculturable microorganisms

Despite the fact that microbiologists, in the early decades of the 20th century, were not aware of the unculturability of several microorganisms and even thought that almost all prokaryotic diversity had been already revealed, recent investigations on microbial biodiversity took a considerable jump forward. The difficulty to cultivate the majority of environmental microorganisms was revealed by the so-called "great plate count anomaly", meaning the evident discrepancy between the numbers of microorganisms estimated by plating and by microscopy (Jannasch and Jones, 1959; Kogure *et al*., 1979; Staley and Konopka, 1985). Indeed, some studies pointed that colony forming units (CFUs) and microscope counts at aquatic environments can differ on four to six orders of magnitude (Grimes *et al*., 1986) while also has been estimated that, in soil, only 0.1 to 1 % of bacteria can be cultivated under laboratory conditions (Torsvik and Ovreas, 2002). The percentage of culturable microorganisms has been estimated for a number of environments (table 2):

Table 2: Culturability determined as a percentage of culturable bacteria (CFU) in comparison with total cell microscopy-based counts (adapted from Amman *et al*., 1995).

Environment	Culturability (%)
Marine	0.001-0.1
Freshwater	0.25
Mesotrophic lake	0.1-1
Unpolluted estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

Accordingly, other studies revealed the viability and importance of the unculturable microorganisms in natural ecosystems (Colwell *et al*., 1996; Marshall *et al*., 1985). Normally, the difficulty to culture the major part of microorganisms results from using laboratory highly artificial and restrictive growth conditions because it is very difficult to mimic the natural conditions (Barer and Harwood, 1999).

Comparing the almost a million recognized insect species and more than 260.000 vascular plants species described and named, the number of prokaryotic species cultured is extremely low. As stated above, the principal reason places on the incapability for developing media to support all different kinds of bacteria growth and metabolism. The researchers still don't have the means or the knowledge to culture and isolate most of bacteria (Fontana *et al*., 2005).

Actually, the number of prokaryotic species may go beyond all other forms of life in our planet and Earth's biomass is mostly composed by prokaryotic cells (Whitman *et al*., 1998; Curtis *et al*., 2002). The about 6600 validly named bacteria (Floyd *et al*., 2005) represent almost a anecdotic number when compared to the estimated diversity of, by instance, 6300 species per gram of soil (Curtis *et al*., 2002). Indeed, several authors had state that is virtually impossible to find out all the microbial species and their functions within a microbial community (Brock, 1987; Wilson, 1994).

Due to the use of methodologies based on phylogenetic analysis of genes encoding the small subunit ribosomal RNA sequences, microbial biodiversity studies have made amazing discoveries. Culture-independent methods have been proposed to provide a less biased picture of the richness of bacterial communities than culture-dependent methods because of the selective pressure imposed by the requirement of the latter for growth on a solid substrate, leading to the isolation of a plate-growth-adapted subpopulation from the communities (Staley and Konopka, 1985; Brock, 1987; Amann *et al.*, 1995).

However, other researchers have a different opinion, affirming that cultivable bacteria may constitute the majority of the total bacterial numbers in samples on the basis of DNA-DNA hybridization between bacterial isolates and community DNA from natural samples (Rehnstam *et al*., 1993; Pinhassi *et al*., 1997).

These two apparently divergent points of view are not automatically mutually exclusive.

Quantification analysis show that in natural samples a good estimative by DNA-DNA hybridization is provided in terms of actively growing and dominant species, while these abundance analysis could not be overlapped with the results obtained from the petri dish media because dominant species growing in the latter might be minorities in natural environments and vice versa (Kisand and Wikner, 2003). Also, it is probable that many species do not succeed to grow in a given media or perhaps show such a few CFU that are

unnoticed in isolation stage. So, culture-dependent methods can miscalculate the community richness while culture-independent methods could detect species that are missed by plating leading to a better diversity evaluation (Kisand and Wikner, 2003).

However, several potential biases exist in culture-independent approaches, mostly because it is dependent on PCR and other molecular techniques. Therefore, several methodological steps such as extraction of DNA from the community, the PCR phase, enzymatic reactions, cloning into vectors and separation of 16S rDNA by chemical or temperature denaturing gradient electrophoresis have its own potential shortcomings (von Wintzingerode *et al*., 1997; Muyzer and Smalla, 1998). Other problems come from theoretical issues such as defining and using significant taxonomic units of diversity (species) (Forney *et al*., 2004).

On the other hand currently available culture-independent techniques cannot assess the immense functional and metabolic diversity owned by the prokaryotic world. Microbes are key players in major geochemical cycles and climate change, and have practical importance in agriculture, disease prevention, animal nutrition, waste treatment, biotechnology and much more. Understanding microbial communities structure and function is crucial to understand biogeochemical cycles that sustain life on Earth and to evaluate the impact of human activities on ecosystems functioning. All the recent discoveries concerning microbial diversity and abundance led Mark Wheelis to state: 'The Earth is a microbial planet, on which macroorganisms are recent additions — highly interesting and extremely complex in ways that most microbes aren't, but in the final analysis relatively unimportant in a global context' (Woese, 1998).

The recently reported high complexity of the ocean metagenome suggests that this ecosystem is certainly even more diverse than previously thought, comprising a deeper genetic reservoir and consequently a huge potential (DeLong, 2004; Venter *et al*., 2004). However, the physiology and ecological roles of many microbes inhabiting aquatic environments remain obscure because they were not yet been brought into culture.

In summary isolation and cultivation techniques remain essential to understand the physiology and ecology of bacteria.

1.2.1 Bacteria identification: molecular evolutionary clocks

The idea that certain macromolecules could be used as molecular chronometers or marker molecules to document the evolutionary history of organisms was first proposed by Zuckerkandl and Pauling in 1965 (Zuckerkandl and Pauling, 1965). A good molecular chronometer is a molecule which is universally present within the group, in order to allow the comparison of organisms; is functionally homologous between individuals and thus show sequence similarities; has a sequence capable of reflecting evolutionary changes and, finally, it must have highly conserved regions for aligning during analyses, which also facilitates primer design (Madigan and Martinko, 2006).

Carl Woese and his colleagues produced seminal studies in which they used the sequence of the 16S rRNA gene to establish phylogenetic relationships between prokaryotes (Woese, 1987). The 16S rRNA gene has several attractive features to be used as molecular marker for the domain Bacteria, such as: it is an housekeeping gene; it is universally present in all bacteria; it is relatively large (~1500bp) providing a great amount of information to address phylogenetic relationships; it is composed in one hand by highly conserved regions and on the other hand by highly variable regions. This last characteristic facilitates the alignment of 16S rDNA sequences and the design of universal and/or specific primers. In addition, public databases are available, in which we can find a large number of sequences (Janda and Abbott, 2007).

Taxonomically, the comparison between 16S rRNA gene sequences allows the affiliation of an organism to a genus and frequently to a species or subspecies. Although, one needs first to agree on a species definition and this is not straightforward for organisms that do not reproduce sexually and show little morphological diversity. Today, the species for prokaryotes is mainly defined on the basis of DNA similarity (Oren, 2004). The question of what a species is or is supposed to be was discussed by a huge quantity of literature, and taxonomic specialists often diverge on how to delimit species within some particular groups (Mallet, 2001).

DNA-DNA hybridization has been considered the reference for prokaryotic species description and remains a frequently applied technique (Ward and Fraser, 2005). Although the relationship is not linear, strains that show DNA-DNA relatedness values greater than 70% tend to have very similar 16S rDNA sequences – similarity above 97%. Even so,

there is no agreement on the precise level of genetic similarity between 16S rDNA sequences that defines a species: 99 to 99.5% is frequently used (Clarridge, 2002). According to Bosshard *et al.*, to define a species, a similarity percentage of 16S rDNA over or equal to 99% is needed, and a genus is identified with a similarity within the range of 95-99% (Bosshard, *et al.*, 2003). Some authors consider that a strain with less than 97% of homology in 16S rRNA gene with his most similar described species can be considered a new species (Staley, 2006).

In summary, 16S rRNA gene is one of the most suitable housekeeping genes to assess valid phylogenetic information and certainly the most widely used (Weisburg *et al.*, 1991).

1.3. Estuaries

Most estuaries are the product of flooding of river valleys during the Holocene when the sea levels rise after the last major glaciations. A variety of adverse climatic and tectonic influence occur so that the timing and rates of change differ according to the geographic location (Pirazzoli, 1991), but in general, increase in sea level and consequent morphological change in estuaries was more rapidly between 10 000 and 5000 years ago (Kennish, 2002; Ridgway and Shimmield, 2002).

Because of its nature and its position between terrestrial and marine environments, they represent important areas between fluvial and marine systems which have been the focal point for a large variety of human activities and have become places of great industrial ports as well as urban and recreational development (Ridgway and Shimmield, 2002).

Estuaries are generally characterized by having very strong biological and chemical environmental gradients resulting from the fresh and marine water mixture (Crump, 2004). So, the salinity gradient, nutrient concentration and the organic composition have significant influence on the prokaryotic structure of these systems (Bouvier and Del Giorgio, 2002; Ducklow and Fasham, 1992). It is known that bacteria play an important role in the food chain and biogeochemical cycles, particularly in organic matter degradation and chemical substances decomposition (Mendo *et al.*, 2003).

Estuarine and marine environments have been reported as major repositories of anthropogenic waste for decades. Contaminants enter estuarine and marine waters via several key pathways, specifically direct pipeline discharges from coastal communities, discharges from ships, riverine input, atmospheric deposition and nonpoint pollution sources from land runoff. The most common anthropogenic wastes disposed in coastal areas are industrial and municipal wastes, sewage sludge and dredged material (Kennish, 2002; Ridgway and Shimmield, 2002)

So, estuaries display an extensive range of human impacts that can compromise their ecological integrity concerning the fast population expansion and uncontrolled development in many coastal regions worldwide (Weber, 1994; Hameedi, 1997; Kennish, 2002).

Long-term environmental problems at estuaries require corrective measures to recover the viability and health of these precious coastal systems. High population densities of microbes, plankton, benthic flora and fauna are characteristic of estuaries; however, these organisms have the tendency to be susceptible to human activities in coastal watersheds and adjoining embayment (Kennish, 2002).

The high primary production and quite large amount of organic matter that accumulate in these systems provides microorganisms, namely bacteria, to accomplish high abundances. Bacterial counts in estuarine waters range from 10^6–10^8 cells ml^{-1}, and they decline seaward (Ducklow and Shiah, 1993; Valiela, 1995; Pinet, 2000).

Attending to the bacterial abundance, diversity and ubiquity, it is fundamental to explore their key functions, i.e., the role played, within biogeochemical processes such as primary production and consumption of organic matter, cycling of nutrient elements, regulation of the atmosphere composition, nitrogen fixation, and photosynthesis (Doney *et al*., 2004).

For estuaries, which may dominate global air–sea exchange of both CH_4 and CO, an improved understanding of community structure both in the SML and subsurface waters is critical (Upstill-Goddard *et al*., 2000; Stubbins, 2001).

1.3.1. 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. 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 (Figure 2), by a channel between Barra and S. Jacinto (Hall *et al*., 1985; Dias *et al*., 1999).

It is a mesotrophic estuarine system with a diverse topography, being separated from the sea by a sandy boundary and presenting a complex net of internal canals (Figure 3). 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).

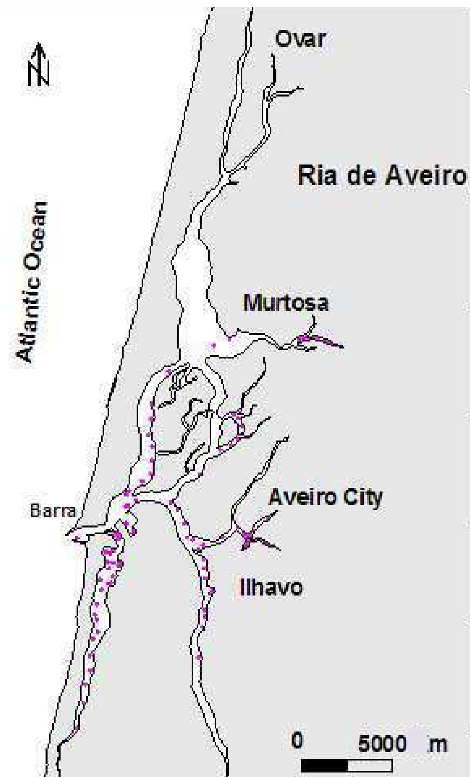

Figure 2: Ria de Aveiro – map.

Figure 3: Estuary' biggest channel - Costa Nova, representing the only communication to the open sea.

Over time, Ria de Aveiro has been the target of several pollutants discharges, being the main sources of contamination the sludge waste from Aveiro's city and the diffuse pollution associated to Aveiro's seaport activities (Figure 4), 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. Only recently efforts have been made to solve the pollution problem in order to recycle and preserve the water quality and the ecosystem health.

Figure 4: Ria de Aveiro's harbour activities.

2. Objectives

The aquatic surface microlayer constitutes a particular and to some extent extreme environment. Considering its distinct physicochemical characteristics it is proper to hypothesize that this environment is probably inhabited by distinct bacterial communities, displaying particular phylogenetic and functional properties. The main aims of the present study were:

- to characterize differences between the SML and UW in terms of abundance of culturable heterotrophic bacteria.
- to determine the bacterial diversity within the bacterial culturable fraction at the SML and to compare this with the diversity of bacteria in the UW.
- to establish a culture collection of strains from the SML and UW to be used in future studies aiming a more detailed characterization of the isolates.

Material and Methods

3. Material and Methods

3.1. Sampling sites

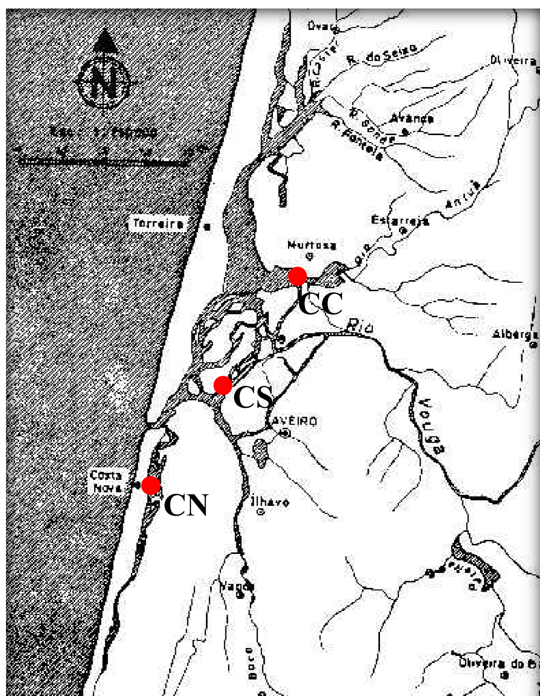

Figure 5: Ria de Aveiro map. CN, CC and CS are signalized.

Our work concerned three sampling sites in the estuary Ria de Aveiro, corresponding to different environmental parameters and exposure to pollutants, namely: Costa Nova (40º 36’ 30.00’’ N, 8º 44’53.54’’ W), Cais do Chegado (40º 43’ 46.70’’ N, 8º 38’ 53.88’’ W) and Cais do Sporting (40º 38’ 26.77’’ N, 8º 40’ 20.29’’ W) that will be referred as CN, CC and CS, respectively (Figure 5).

CN is located near the only communication with the open sea. The sampling site was located near a small port of motor boats and the main sources of contamination are diffuse domestic sewage inputs and run-off from agriculture fields. CC is highly impacted by industrial effluents, namely by effluents from chemical industrial plants located in its vicinity. The sampling site was also located near a small port of fishing boats. CS is located near the city of Aveiro and is subjected to anthropogenic pressure mainly by the presence of harbor facilities and urban effluents.

3.2. Sampling procedure

Five campaigns were performed in May 5th, May 21st, June 18th, September 9th and September 29th. Campaigns will subsequently be designated C1 (May 5th), C2 (May 21st), C3 (June 18th), C4 (September 9th) and C5 (September 29th), respectively. For each sampling site samples from SML and UW were collected always at low-tide in two distinct periods: day (maximum light) and night (minimum light).

The SML samples were obtained by the sampling method Glass-Plate (Harvey and Burzell, 1972). Briefly, a glass plate (dimensions: 0.25 m wide x 0.35 m long and 4 mm thick) was rinsed with ethanol, sterile distilled water and several times with water from the sampling site. Consecutively, it was vertically immersed into the water column and then lifted up and left still for fifteen seconds to allow the exceeding water to runoff. The remaining adherent water was recovered by introducing the plate between two Teflon sheets and collected into a sterilized bottle (Figure 6). The UW samples were recovered into a sterilized bottle from a depth of about 0.5m. The bottle was opened and closed underwater to minimize SML interference (Figure 7).

Figure 6: Performing Glass-Plate sampling, SML.

Figure 7: Performing UW sampling.

Temperature and salinity were determined using a WTW LF 196 Conductivity Meter.

3.3. Samples processing

Samples were kept cold in a thermal container during transportation from the field to the laboratory where they were processed within the next four hours.

CFUs were counted as visible colonies on agar plates obtained by plating 100 µl of the appropriate dilutions prepared in sterile Ringer's solution. Four replicates for each sample were counted after 3 and 5 days of incubation at 25°C in the dark.

Resistant bacteria were quantified on samples collected at night, during campaigns C4 and C5, by following the same procedure and media supplemented with ampicillin (50

μgml^{-1}) and tetracycline (30 μgml^{-1}). A stock solution of both antibiotics (100 $mgml^{-1}$) was prepared and sterilized by filtration using a filter of 0.2 µm (Orange Scientific - Braine-l'Alleud, Belgium) and then stored at -20ºC.

3.3.1. Culture media

Two culture media were chosen: ***Pseudomonas Aeromonas*** **Selective Agar Base (GSP - MERCK) and Estuarine Agar (EA- Difco)**. The composition for 1 liter is given below. Immediately after preparation, according to manufacturer's information, the media were sterilized for fifteen minutes at 121ºC.

Composition (g/litre):

***Pseudomonas Aeromonas Selective Agar Base (GSP - MERCK, Darmstadt, Germany)**:*

Sodium L(+)glutamate 10.0 g; starch, soluble 20.0g; potassium dihydrogen phosphate 2.0g; magnesium sulfate 0.5g; phenol red 0.36g; agar 12.0g.

Estuarine Broth (EB – Difco, Voigt Global Distribution):

Peptone 5.0 g; Yeast Extract 1.0 g; Ferric Citrate 0.1 g; Sodium Chloride 19.45 g; Magnesium Chloride 5.9 g; Magnesium Sulfate 3.24 g; Calcium Chloride 1.8 g; Potassium Chloride 0.55 g; Sodium Bicarbonate 0.16 g; Potassium Bromide 0.08 g; Strontium Chloride 34.0 mg; Boric Acid 22.0 mg; Sodium Silicate 4.0 mg; Sodium Fluoride 2.4 mg; Ammonium Nitrate 1.6 mg; Disodium Phosphate 8.0 mg.
The composition of the medium Estuarine Agar (EA – Difco) was identical to Estuarine Broth supplemented with 12.0g of agar.

***Luria-Bertani (LB- MERCK, Darmstadt, Germany)**:*

Yeast extract 5.0g; peptone from casein 10.0g; sodium chloride 10.0g.

3.4. Strains isolation

Colonies with distinct morphologies on the spread plates from C1, C2 and C3 were purified on the same media (Figure 8). Isolates were stored at −80 ºC in 96-well microplates with culture media plus 15% glycerol.

We assigned a quadruple code for each strain, attending to the media from where it was selected (GSP or EA – using the first letter from each acronym – G or E) as well as the sampling site (CN, CC or CS – using the last letter from each acronym – N, C or S), type of sample (SML or UW, BN and BP respectively – using the last letter from each – N or P) and campaign number (C1, C2 or C3 - I, II and III respectively).

So, for example GNP7 III means that this isolate was selected from a GSP medium plate, from the sampling site CN and represents a UW (BP) sample obtained at C3- III. As said before several isolates were selected from the same sample so before the campaign designation the code has also the isolate number (from 1 to 10).

Figure 8: Example of a GSP medium plate with colonies displaying different morphologic characteristics.

3.5. Strains identification

3.5.1. DNA extraction

To obtain DNA from the bacterial isolates 5 different strategies were evaluated:

1) 1µl of an overnight liquid culture was directly added to the PCR reaction.

2) Overnight growth colonies were picked with a sterile toothpick, suspended in 5 µl sterile water and the mixture was incubated for 10 min at 100 ºC. 3 µl of the lysate obtained was added to the PCR reaction.
3) 10 µl of an overnight liquid culture was centrifuged at 13.400 rpm for 3min the supernatant was removed. The pellet was ressuspended in 10 µl of sterilized water which was incubated for 10 min at 100ºC. 3 µl of the lysate obtained was added to the PCR reaction.
4) 5 µl of an overnight liquid culture was incubated for 10 min at 100 ºC. Centrifuged at 13.400 rpm for 30 seconds. 3 µl of the lysate obtained was added to the PCR reaction.
5) The Genomic DNA Purification Kit from MBI Fermentas (Vilnius, Lithuania) was used according to the manufacturer's instructions with slight modifications as described below.

Genomic DNA Purification Kit

- Strains were grown overnight in LB broth;
- 1 ml of cell culture was centrifuged during 5 minutes at 13.200 rpm and the pellet was ressuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0);
- 25 µl of 10 mg/ml lysozyme solution (Eurobio, France) were added and the suspension was incubated for 1 hour at 37ºC to improve lysis;
- The suspension was mixed with 400 µl of lysis solution (Genomic DNA Purification Kit) and the mixtures were incubated for 10 minutes at 65ºC;
- Immediately, 600 µl of chloroform were added followed by softly inversion in order to emulsify the mixture;
- The sample was centrifuged at 13.400 rpm during 10 minutes;
- The top aqueous phase which contained the DNA was transferred to a new tube and the last two steps were repeated;
- Following, 0.6 volumes of isopropanol were added to allow the DNA precipitation, and solution was gently inverted and incubated at 4ºC during 10 minutes;
- The mixture was then centrifuged at 13.400 rpm during 15 minutes;

- The supernatant was removed and the pellet was completed dissolved in 100 µl of 1.2 M NaCl solution;
- 250 µl of cold ethanol were added and DNA was left to precipitate at -20ºC during 45 minutes;
- The mixture was centrifuged during 15 minutes at 13.200 rpm;
- The supernatant was eliminated and the pellet was washed with 70% ethanol;
- The DNA was ressuspended in 50 µl of TE and stored at -20ºC.

3.5.2. Amplification of 16S rDNA

For 16S rRNA gene amplification the following bacterial universal primers were used (table 3):

Table 3: Forward and reverse primers sequence

	Designation	Sequence
Forward primer	27 F	5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse primer	1492 R	5'-GGTTACCTTGTTACGACTT-3'

The reactions were performed in a Bio-Rad My Cycler ™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The Taq polymerase, the buffer and the dNTP's were from MBI Fermentas (Vilnius, Lithuania).

Each reaction mixture (25 µl) contained:

- 1 x PCR buffer
- 3 mM $MgCl_2$
- 5 % dimethylsulfoxide
- 200 mM of each nucleotide
- 7.5 pmol of each primer

- 0.5 U *Taq polymerase*
- 50-100 ng of DNA.

Amplification conditions and expected fragment length are listed in the table below (table 4):

Table 4 Detailed PCR program

<table>
<tr><th>Gene target</th><th colspan="2">Amplification conditions</th><th>Expected fragment lenght</th></tr>
<tr><td rowspan="5">16S rDNA</td><td>1 cycle</td><td>Initial denaturation: 93ºC for 3 min</td><td rowspan="5">1500 bp</td></tr>
<tr><td rowspan="3">35 cycles</td><td>Denaturation: 94ºC for 1 min</td></tr>
<tr><td>Annealing: 51ºC for 2 min</td></tr>
<tr><td>Extension: 72ºC for 2 min</td></tr>
<tr><td>1 cycle</td><td>Final extension: 72ºC for 10 min</td></tr>
</table>

To analyze the resulting amplicons, 5 µl of PCR products were loaded in 1% agarose gels in 1x TAE buffer (Bio-Rad, Hercules, CA, USA) along with the molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus 0.1 µg/µl (MBI Fermentas, Vilnius, Lithuania). Electrophoresis was performed at 80V during 80 minutes. The gels were stained in ethidium bromide and then rinsed in distilled water during 5 minutes. Images were acquired using the G:BOX system (Syngene, Cambridge, UK).

3.5.3. Amplified ribosomal DNA restriction analysis (ARDRA)

A preliminary discrimination of bacterial isolates was performed by ARDRA, a simple method based on restriction digestion of the amplified bacterial 16S rDNA.

The endonuclease enzyme *Hae*III was chosen because it has a high cutting frequency (table 5).

Table 5: Recognition site of endonuclese *Hae*III.

***Hae*III recognition site**	5'- ...GG↓CC ...- 3'
	3'- ...CC↑GG ...- 5'

Restriction reactions were prepared according to the following recipe:

Sterilized water	7.8 µl
Enzyme buffer	2 U/µl
*Hae*III	0.2 U/µl
PCR product	10 µl

The reaction was incubated at 37ºC for sixteen hours. To analyze the resulting restriction profiles 10 µl of the restriction reaction were loaded into 1.5% agarose gels in 1x TAE buffer (Bio-Rad, Hercules, CA, USA). Two lanes in each gel were loaded with a molecular weight marker GeneRuler ™ DNA Ladder Mix – 0.5 µg/µl (MBI Fermentas, Vilnius, Lithuania) was included. Electrophoresis was performed at 80V during 80 minutes. The gels were stained in ethidium bromide and then rinsed in distilled water during 5 minutes. Images were acquired using the G:BOX system (Syngene, Cambridge, UK).

Gels images were analyzed with the GelCompar II software (Applied Maths, Kortrijk, Belgium).

3.5.4. Sequencing and sequence analysis

From each ARDRA profile identified during gel analysis, at least two representatives were selected for sequencing analysis.

3.5.4.1. Purification of DNA products for subsequent sequencing

PCR products were purified using the JETQUICK PCR Product Purification Spin Kit (Genomed, LOhne, Germany) according to the manufacturer instructions.

Detailed procedure:

- Four hundred µl of Solution H1 (JETQUICK KIT) were added to the PCR product;
- A JETQUICK spin column was placed into a 2 ml receiver tube and the previous mixture was loaded into it;
- The column was centrifuged at 12.000 x g during one minute;
- The flow through was discarded and the column was placed again in the receiver tube;
- The column was centrifuged again at the maximum speed for 1 minute;
- To elute the DNA the JETQUICK spin column was placed into a 1.5 ml microtube and 50 µl of sterile water were added onto the center of the silica matrix of the column;
- The column was centrifuged at 12.000 x g during two minutes to collect the purified PCR product.

3.5.4.2. Sequencing and sequence analysis

Purified PCR products were used as templates in sequencing reactions that were carried out by the company STAB-VIDA (Oeiras, Portugal). Sequences were visualized with the FINCH software Version 1.4 (Geospiza's GeneSifter Lab Edition - http://www.geospiza.com/Products/finchtv.shtml) and manually edited. 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.

Sequence data from the 16S rRNA gene were the basis for the phylogenetic comparison of BN and BP. When comparing sequences, we used the common criterion in which 16S rDNA sequences that are above or equal to 97% similar are defined as the same species (Staley, 2006).

3.5.5. Phylogenetic analysis

Sequences obtained during this study and sequences from reference taxa retrieved from the GenBank database were aligned using the CLUSTAL X program (Thompson, *et al*, 1997). Phylogenetic analysis were performed with PAUP* version 4.0b10 (Swofford, 2003). Trees were produced using the neighbour-joining method. Bootstrap support values (1000 replicates) were calculated.

3.6. Statistical analysis

Statistical significance of the CFUs enumeration was assessed using the t-test from the software package SPSS version 16 (SPSS Inc, Chicago, Illinois).

Results and Discussion

4. Results and Discussion

4.1. Measurements of physical parameters

Salinity values were highly variable and ranged from 3,8 to 33,3 for site CS, from 1,8 to 30,1 for site CC and from 3,9 to 27,9 for site CN. The sampling site with the highest salinity values in each campaign was CS. In contrast, CC had the lowest salinity values in the first two campaigns (CS>CN>CC) but in third and forth campaign the lowest salinity values were registered in CN (CS>CC>CN). Salinity values generally increased from May to September.

Temperature ranged from 16 ºC to 20,8 ºC for site CS, from 15,9 ºC to 21,7 ºC for site CC and from 16 ºC to 20,6 ºC for site CN; the lowest values were registered during C1 and were rather stable for the remaining campaigns (Annex 1).

4.2. Enumeration of culturable bacteria

The enumeration of culturable bacteria was performed for five campaigns: C1, C2, C3, C4 and C5. Graphics 1-5 (figure 9) present the mean values of CFUs for each campaign. Higher values were always registered for SML samples when compared with samples from UW (Figure 9) and most of the times these differences were statiscally significant. In fact higher CFU values for SML samples were registered both in GSP and EA plates, for the three sampling sites and independently of the sampling moment (day vs. night) and of the campaign (C1 to C5). Considering the sum of all samples total CFUs/ml in SML plates was approximately $47x10^3$, versus $13x10^3$ for UW ($p<0.00001$).

It has been demonstrated by other studies that larger numbers of bacteria are found at water, whether saline or freshwaters, surfaces microlayers (Zavarzin, 1955; Dratchev *et al*., 1957; Bogorov, 1966; Harvey, 1966; Babenzien & Schwartz, 1970; Morita and Burton, 1970; Sieburth, 1971; Tsyban, 1971; Hatcher & Parker, 1974; Sieburth *et al.*, 1976; Dutka & Kwan, 1978; Crawford *et al*., 1982; Danos et al., 1983; Münster *et al*., 1998). Several reports suggest that bacteria may be 10^2–10^5 times more abundant in the SML than in

subsurface waters (Bezdek & Carlucci, 1972; Sieburth *et al*., 1976). Even so, occasionally, lower microbial abundances were also reported for the SML (Bell and Albright, 1982) and Agogué and colleagues reported statistically non-significant differences between SML and UW abundances (Agogué *et al*., 2005).

First of all, this variability in microbial abundances reported in the literature might be related to the use of different sampling devices to collect the SML and/or to natural ecological variability of the enrichment (Carlson, 1982; Hardy, 1997; Agogué *et al*., 2004).

One of the explanations proposed for the fact that SML generally has a higher quantity of bacteria is that this layer accumulates organic matter, increasing the nutrients concentrations, and therefore the availability of carbon sources for heterotrophic bacteria (Zdanowski & Figueiras 1999).

The electrostatic interactions between viable bacteria and rising particles explained by a bubble flotation can also lead to a higher abundance of bacteria in the SML. This happens because living bacteria have a negative charge that, attending to the higher seawater pH, results in a passively passage by attracting cations (Grasland *et al*., 2003).

From the graphics presented in Figure 9 and Figure 10, information on the spatial and temporal variability of SML and UW communities can also be retrieved. In CN, the higher values for SML were registered in C4 (September) and considerable lower values for UW samples were registered in C3 (June) and C4 (September). In CC, CFU values were rather stable between campaigns but the highest BN values and the lowest BP values were registered in C4. In CS the highest BN levels were registered in C1 and the lowest BN and BP values were registered in C4. In the first three campaigns, BN increases from CN to CS. However, this tendency was not registered in the last two campaigns. We can speculate that the seasonal variations may play a role, as the first three campaigns were performed in spring and the last two in the end of the summer. The variability of the UW cultivable bacterial abundance was lower between sampling sites. Overall, CN had the highest abundance of culturable bacteria: the sum of SML and UW counts was $37x10^3$ against $26x10^3$ in CC, the site where total counts were lower. However differences were not statistically significant ($p=0.149$) (Figure 10).

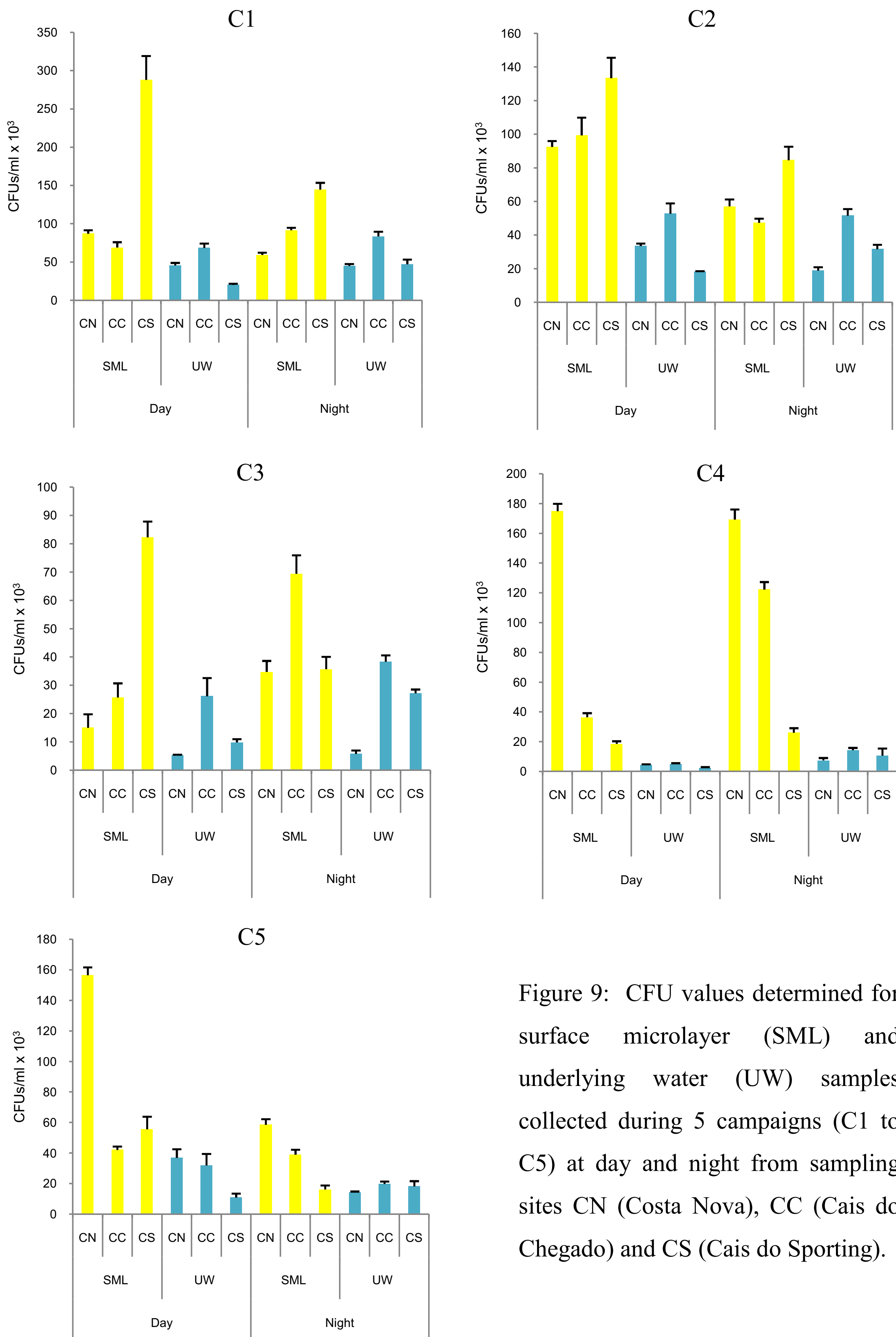

Figure 9: CFU values determined for surface microlayer (SML) and underlying water (UW) samples collected during 5 campaigns (C1 to C5) at day and night from sampling sites CN (Costa Nova), CC (Cais do Chegado) and CS (Cais do Sporting).

In summary, the extent of differences between SML and UW counts were frequently dependent on the sampling site and on the sampling moment. For example in C1, SML counts for samples collected during day at CS were considerably higher when compared to SML counts for the other sampling sites. However, the UW samples collected at the same site and at the same moment displayed considerably lower values of CFUs when compared to the other two sampling sites. Other examples can easily be observed in Figure 9. Our results suggest that the temporal and seasonal factors probably play an important role in defining the extent of differences on abundance between the SML and UW culturable communities.

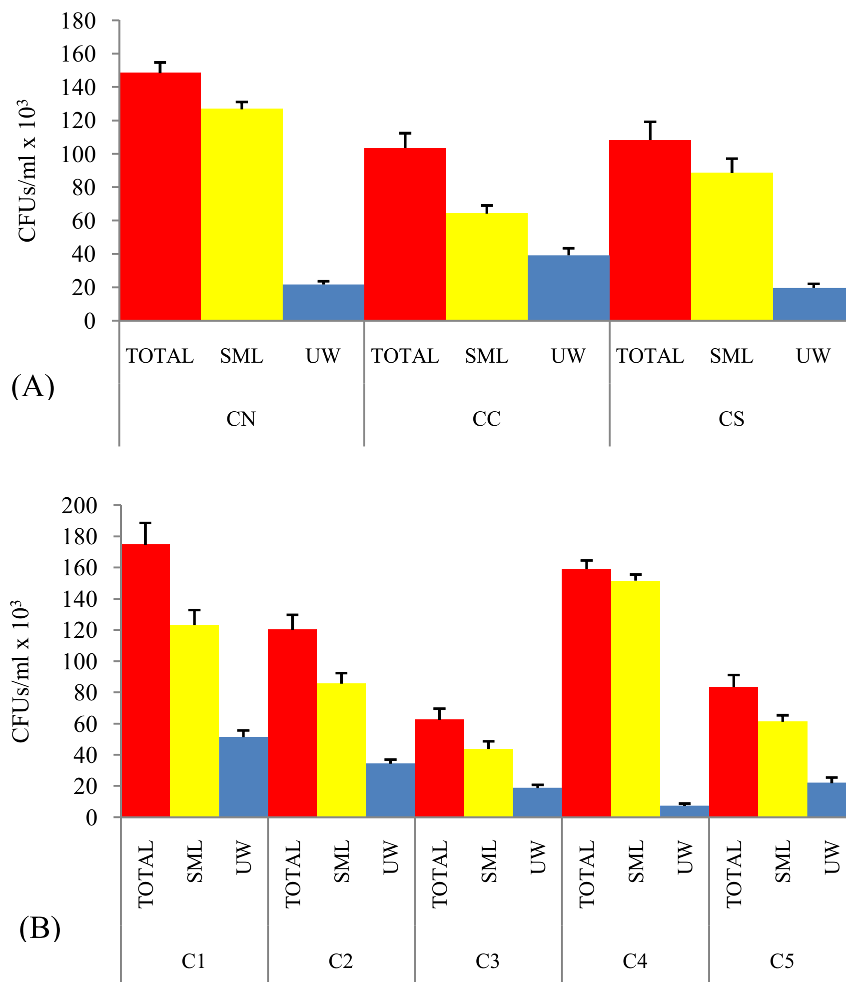

Figure 10: Graphics show the total sum of CFU values for sampling sites CS (Cais do Sporting), CN (Costa Nova) and CC (Cais do Chegado) (A) and for campaigns C1 to C5 (B). SML-surface microlayer; UW-underlying water.

When we first designed our work we raised the possibility that total populations of bacteria may change regarding sun exposure. With our results (figure 11), it is notorious that in most cases bacterial abundance within the SML suffers a significant decline from day to night (p=0.029). Probably sun exposure is not the only variable responsible for this decline; other parameters like temperature and salinity might be involved. So, we have to be cautious in concluding that the lower abundances of SML bacteria at night are just mainly due to sun exposure effects. Bacterial abundance within UW increases from day to night. However differences were not statistically significant (p=0.423). Bacteria from the SML possibly migrate in the night toward UW. A few previously conducted studies indicated that SML bacterial population size and activity varied according to diel cycles (Sieburth *et al*., 1983; Maki & Remsen, 1989). Hermansson & Dahlback also reported the migration of active bacteria from the SML into the UW (Hermansson & Dahlback, 1983). This circadian rhythm deserves further attention and must be considered when characterizing SML and UW populations.

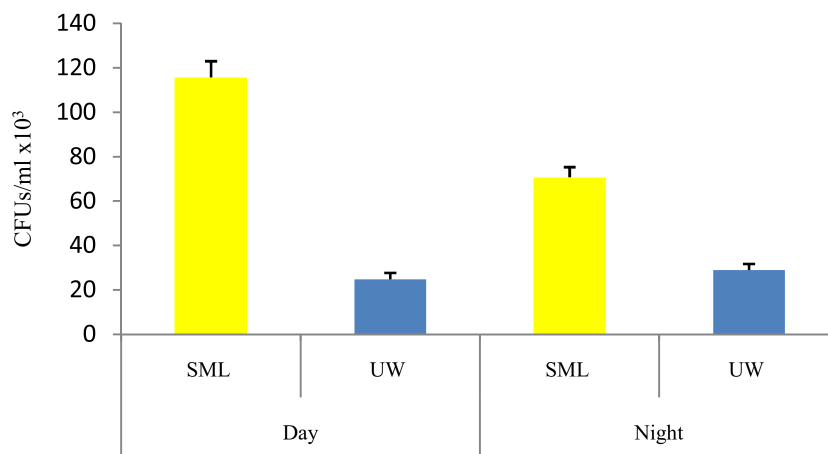

Figure 11: Mean counts joining all the campaigns and sampling sites in order to enhance the day/night variations between both layers.

4.3. Enumeration of culturable bacteria in media supplemented with antibiotics

Resistant bacteria were quantified for SML and UW samples collected at night in campaigns C4 and C5. Results are shown in figure 12. Total counts of CFUs were lower in Tetracycline plates than in Ampicillin plates. Making a comparative analysis of this results, one must cross the obtained results from media supplemented with antibiotic with the obtained results from the same samples but plated on media without antibiotic. So, analyzing the number of CFUs discrepancy between both layers, CFUs in SML were 10 times more abundant than in UW at C4 and 2 times more abundant at C5, on media without antibiotics. Comparing these proportions with the numbers obtained on media supplemented with antibiotics (for the same samples), the following results were obtained:

- For ampicillin selection on campaign C4, for SML only 4.5% of the CFUs number estimated in plates not supplemented with antibiotic have grown, while for UW the decrease was not so dramatic but yet only 8% have resisted.
- For tetracycline selection on C4, for SML just 0.49% of the CFUs number estimated on plates without antibiotic formed colonies, while for UW the percentage was, again, higher – 3.8% have grown.
- For ampicillin selection on C5, for SML only 4% of the CFUs number estimated in plates not supplemented with antibiotic have grown, while for UW the decline was not so sharp but yet only 5.6% have resisted.
- For tetracycline selection on C5, for SML just 1% of the CFUs number estimated on plates not supplemented with antibiotic formed colonies, while for UW the percentage was, again, higher - 3% have grown.

Thus, bacteria belonging to the UW tend to be more resistant to both antibiotics at both campaigns. As SML is an environment with higher levels of pollutants, bacteria inhabiting was expected to be more resistant to antibiotics than bacteria from UW. Studies on this topic are needed namely the estimation of resistant bacteria from both layers during the day period.

Resistance to commonly used antimicrobial agents is among the recognized prokaryotic hazardous characteristics representing potential risk for human health. One of the pointed reasons for increased severeness, longer, more expensive and difficult treatments for infectious diseases are the antibiotic resistance dissemination (French,

2005). We must highlight the fact that most of the studies concerning bacterial antibiotic-resistance and the underlying resistance mechanisms mostly focused clinical settings (French, 2005); however, it has been recognized that natural environments can constitute important reservoirs for antibiotic resistant microorganisms and resistance genetic determinants (Alonso *et al*., 2001; Kümmerer, 2003). Antibiotics are released into the environment through wastewater effluents and agricultural runoffs leading to increasing environmental selective pressures which subsequently lead to adaptation of microorganisms through the development and dissemination of resistance to antibiotics. Aquatic environments are primary receptors for these inputs and also are main locals for dissemination of genetic material between bacteria since water facilitates these phenomena (Chee-Sanford *et al*., 2001; van Elsas and Bailey, 2002). To assess the extent of the potential risk to human and ecological health a better characterization of these reservoirs is strongly needed.

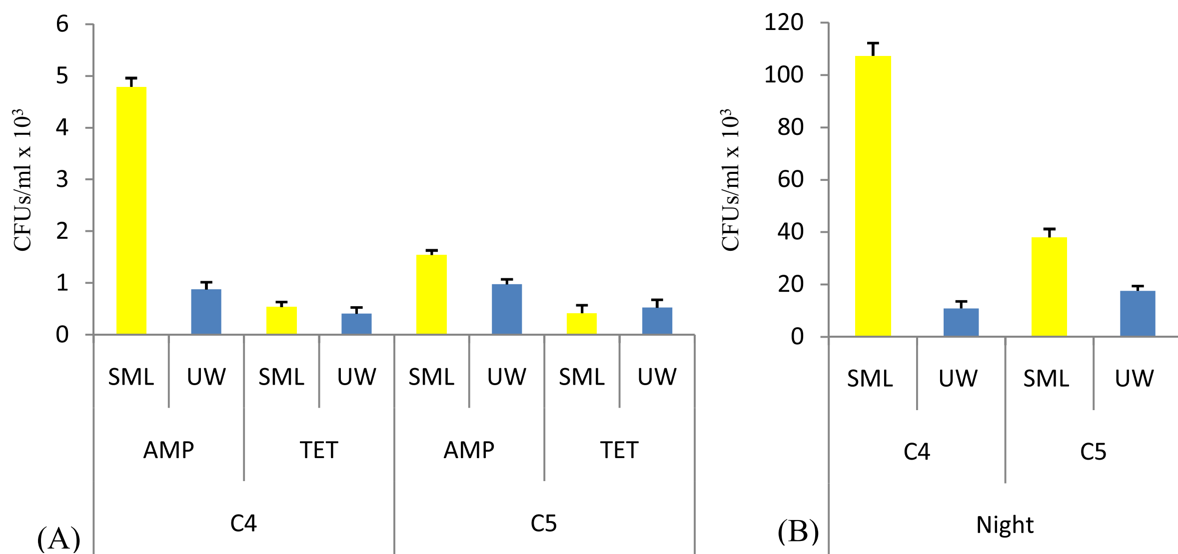

Figure 12: CFU values determined on media supplemented with ampicillin (AMP) and tetracycline (TET) for SML (surface microlayer) and UW (underlying water) samples collected at night during campaigns C4 and C5 (A) and CFUs values for the same period and samples determined on plates without antibiotic (B).

4.4. Phylogenetic diversity

4.4.1 General structure of bacterial communities

To explore if bacterial communities inhabiting both the surface microlayer and underlying waters were consistently different in terms of phylogenetic diversity, 402 bacterial isolates from SML (n=198) and UW (n=204), collected during C1, C2 and C3 from GSP and EA plates, were characterized. Briefly the DNA was obtained from each isolate by using one of five strategies [see material and methods and (Figure 13)] and the 16S rRNA gene was amplified by using universal bacterial primers. The amplicons were discriminated by ARDRA (Figure 14) and at least two representatives of each distinct profile were selected for sequencing analysis.

Seventy-two different profiles were identified. From those 21 profiles were exclusive from SML samples and 28 were exclusive from UW samples (table 7 - Annexes).

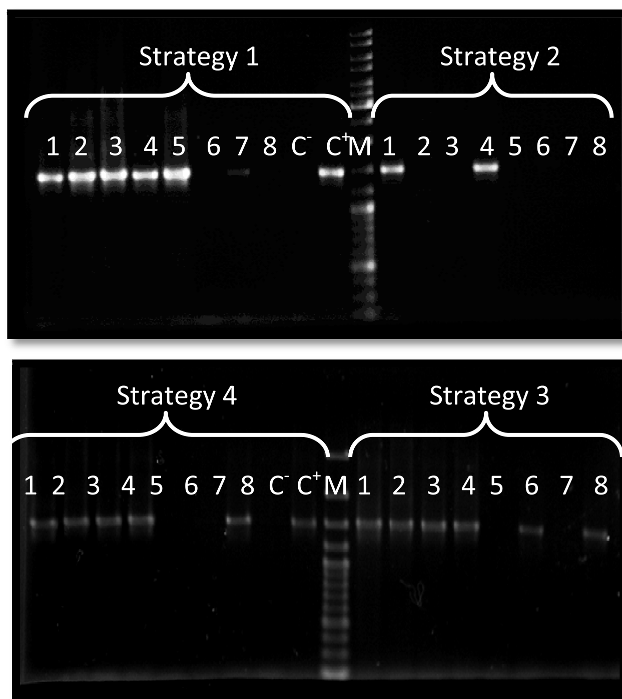

Figure 13: Example of agarose gels showing 16S rDNA fragments amplified using total DNA obtained by several different strategies that are referred in the Material and Methods (see point number 2.5.1. The molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus 0.1 µg/µl (MBI Fermentas, Vilnius, Lithuania) was also included.

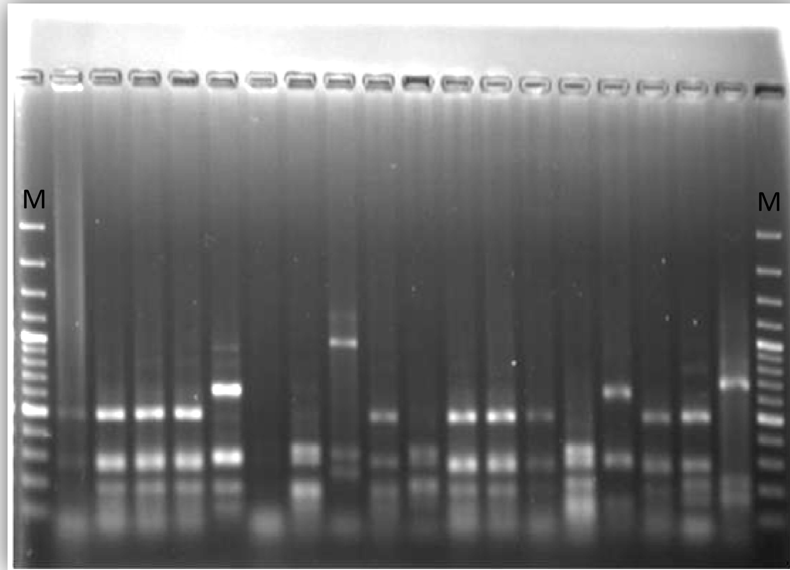

Figure 14: Patterns obtained from the restriction of the 16S rDNA amplicons with the endonuclease enzyme *Hae*III according to procedure explained at Material and Methods (see above point 3.5.4.).

4.4.2. Taxonomic affiliation of bacterial isolates

The obtained nucleotide sequences from each different profile were compared to the GenBank nucleotide data library using the BLAST software to determine their closest phylogenetic relatives. Sequencing results allowed identifying bacteria belonging to 5 different Phyla and 9 different Classes.

Similarity values for the 16S rRNA gene sequences obtained during this study with previously reported sequences in databases as well as the identification of the closest relatives are presented in Table 6. Isolates with sequence similarities >97% to already reported 16S rRNA gene sequences was predominant. Only two isolates showed similarities below 97% (both from UW samples). Isolates with sequence similarities >97% are commonly considered to be representatives of the same species and with similarities between 93% and 97% are probably representatives of the same genus but different species (Staley, 2006). Our results contrast with the study previously reported by Agogué and colleagues in which several isolates displayed similarities below 97%, being seven

recovered from SML sample - providing new putative neustonic isolates (Agogué *et al.*, 2005).

Table 6: Phylogenetic affiliation of bacterial 16S rRNA gene sequences obtained from Ria de Aveiro estuary SML and UW samples.

Code	Closest BLAST match (Accession no.)	Identity %	Origin	Taxonomy
1 GNN1 III	*Psychrobacter fisheri* AB453700.1	99	Oxidative environment	*γ-proteobacteria*
1a GSN5 III	*Psychrobacter* sp. AM403661.1	99	Antarctica soil	*γ-proteobacteria*
1b ESP9 I	*Psychrobacter* sp. AM403661.1	99	Antarctica soil	*γ-proteobacteria*
1c ENP6 II	*Psychrobacter* sp. AM403661.1	99	Antarctica soil	*γ-proteobacteria*
1d GNN8 III	*Psychrobacter fisheri* AB453700.1	99	Oxidative environment	*γ-proteobacteria*
2 ESP7 II	*Deinococcus radiopugnans* Y11334.1	99	Marine	*Deinococcus*
2a GSN9 I	*Deinococcus radiopugnans* Y11334.1	99	Marine	*Deinococcus*
3 ESP3 II	*Roseivirga ehrenbergii* AY739663.1	99	Sea water	*Sphingobacteria*
4 GSP8 II	*Psychrobacter nivimaris* EU880519.1	99	Benthal sediment	*γ-proteobacteria*
4a GSP2 II	*Psychrobacter nivimaris* EU880519.1	99	Benthal sediment	*γ-proteobacteria*
5 ESP4 II	*Cyclobacterium amurskyense* FJ229465.1	100	Intertidal sand film	*Sphingobacteria*
5a ECP6 III	*Cyclobacterium amurskyense* FJ229465.1	100	Intertidal sand film	*Sphingobacteria*
6 ECN8 I	Artic sea ice bacterium AF468359.1	98	Arctic sea ice-melt pond	*α-proteobacteria*
7 GSN5 II	Uncultured bacterium FM873287.1	100	Floor dust	*γ-proteobacteria*
7a GCN9 II	Uncultured bacterium FM873287.1	100	Floor dust	*γ-proteobacteria*
8 GCN4 I	Uncultured bacterium EU468035.1	99	Cheetah feces	*β-proteobacteria*
9 GCN9 I	*Aerococcus piscidermidis* EU376006.1	100	*Sparasoma viridae*	*Bacilli*
9a GSN1 II	*Aerococcus piscidermidis* EU376006.1	100	*Sparasoma viridae*	*Bacilli*
10 GCP8 II	Uncultured bacterium EU104199.1	100	Activated sludge	*γ-proteobacteria*
11 ENP10 III	Uncultured bacterium EU104070.1	98	Activated sludge	*Flavobacteria*
11a ECP7 III	Uncultured bacterium EU104070.1	98	Activated sludge	*Flavobacteria*
12 ENP2 III	*Jannaschia sp.* EU930869.1	97	Sea water	*α-proteobacteria*
13 ENP4 III	*Leeuwenhoekiella aequorea*	98	Polar seas	*Flavobacteria*

		AJ278780.1			
14	ENP5 III	*Agrococcus* sp. EU374908.1	99	Polluet sand	*Actinobacteria*
15	ECP7 III	*Cyclobacterium* sp. AY259502.1	98	Salt marsh sediment	*Sphingobacteria*
16	ESP8 II	*Agrococcus* sp. EU374908.1	99	Polluet sand	*Actinobacteria*
17	GNP6 II	*Marinobacter marinus* AF479689.1	100	Marine	*γ-proteobacteria*
17a	GCP2 II	*Marinobacter marinus* AF479689.1	100	Marine	*γ-proteobacteria*
18	ECP10 I	*Algoriphagus* sp. EU313811.1	97	Daging reservoir water	*Sphingobacteria*
19	ESP5 I	*Agrococcus* sp. EU374908.1	99	Polluet sand	*Actinobacteria*
20	ESN8 III	*Alteromonas macleodii* AB238950.1	99	Sea water	*γ-proteobacteria*
20a	ESP5 III	Uncultured bacterium EU795208.1	99	Station ALOHA	*γ-proteobacteria*
21	GCN6 III	Uncultured *Arcobacter* sp. EF419216.1	98	Estuarine microbiota	*ε-proteobacteria*
22	ESP5 III	*Micrococcus* sp. FJ607363.1 *Citrococcus* sp. FJ607345.1	99	Arsenic-contaminate mine	*Actinobacteria*
22a	ESP6 II	*Micrococcus* sp. FJ607363.1 *Citrococcus* sp. FJ607345.1	99	Arsenic-contaminate mine	*Actinobacteria*
23	ESN7 I	*Bacillus weihenstephanensis* CP000903.1	98	Marine muddy sediment	*Bacilli*
24	GCNP1 II	*Brevundimonas bullata* AJ717390.1	100	Nonsaline alkaline environment	*α-proteobacteria*
25	GSN4 I	Uncultured bacterium AM697264.1 *Stenotrophomonas* sp. AY131216.1	99	Sewage sludge	*γ-proteobacteria*
26	ECP1 II	*Hahella chejuensis* CP000155.1	99	Marine	*γ-proteobacteria*
27	ENN8 I	*Cyclobacterium sp.* EU880511.1	98	Benthal sediment	*Sphingobacteria*
28	ECP6 I	Uncultured gamma proteobacterium AJ301569.1	99	Uranium mining waste piles	*γ-proteobacteria*
29	ECN7 I	*Pseudomonas fluorescens* EF690400.1	100	Plant root	*γ-proteobacteria*
30	GSN8 III	*Pseudoalteromonas* sp. FJ404721.1	99	Sea sediment	*γ-proteobacteria*
31	ESP5 I	*Agrococcus sp.* EU374908.1	99	Hydrocarbon polluted sand	*Actinobacteria*
32	GSP8 II	Psychrobacter sp. FJ457285.1	99	Jellyfish	*γ-proteobacteria*
33	ENN7 III	*Alteromonas macleodii* AB238950.1	99	Sea water	*γ-proteobacteria*
34	GCP10 III	*Acinetobacter johnsonii* AB099655.1	99	Sewage activated sludge	*γ-proteobacteria*
35	GSN6 III	*Pseudomonas stutzeri*	99	Oil reservoir	*γ-proteobacteria*

		U25431.1			
36	ENP10 III	*Flavobacterium* sp. AY145539.1	98	Estuary	*Flavobacteria*
37	ECP9 III	*Shewanella putrefaciens* AB208055.1	99	Marine	*γ-proteobacteria*
38	ESN4 II	*Olleya marilimosa* FJ015035.1	100	Turbot larval rearing unit	*Flavobacteria*
39	ENP5 II	*Psychroserpens* sp. DQ073103.1	99	Marine	*Flavobacteria*
40	ESP3 I	*Pseudoalteromonas* sp. AM913917.1	99	Marine	*γ-proteobacteria*
41	GSP3 II	*Psychrobacter glacincola* EF640972.1 *Psychrobacter aquimaris* EF101547.1	100	Marine	*γ-proteobacteria*
42	ENP6 III	*Erythrobacter sp.* EF512736.1	99	Marine	*α-proteobacteria*
43	ENN3 II	*Cobetia marina* AM945674.1	99	Multipond solar saltern	*γ-proteobacteria*
44	GSN9 II	Uncultured *Alcaligenes* sp. DQ168833.1	100	Sludge	*β-proteobacteria*
45	ENN10 II	*Psychrobacter faecalis* EU370413.1	100	Cow manure	*γ-proteobacteria*
46	GCP3 III	Uncultured bacterium EU104199.1	98	Activated sludge	*γ-proteobacteria*
47	GNN5 III	Uncultured bacterium EU431705.1	97	Percolating waters	*Flavobacteria*
48	GNN5 I	*Shewanella baltica* CP000891.1	100	Marine	*γ-proteobacteria*
48a	ECP10 III	Shewanella sp. AY536556.1	99	Estuarine environment	*γ-proteobacteria*
48b	ECP3 III	Shewanella sp. EU979479.1	99	Columbia River Estuary	*γ-proteobacteria*
49	GSP3III	*Pseudorhodobacter incheonensis* DQ001322.1	99	Marine biofilm	*α-proteobacteria*
50	ENP8 II	*Loktanella atrilutea* AB246747.1	99	Seawater	*α-proteobacteria*
51	GCN9III	*Pseudomonas poae* EU111704.2 *Pseudomonas trivialis* AJ492831.1	100	Phyllosphere	*γ-proteobacteria*
52	GSN1 II	*Pseudomonas graminis* Y11150.1	100	Grasse	*γ-proteobacteria*
53	ESP1II	*Alteromonadaceae* AM913910.1	96	Baltic sea	*γ-proteobacteria*
54	GSN2 III	*Pseudomonas cf. stutzeri* AJ244724.1	99	Oil degrading bacterial consortiun	*γ-proteobacteria*
54a	GNN9III	*Pseudomonas cf. stutzeri* AJ244724.1	99	Oil degrading bacterial consortiun	*γ-proteobacteria*
55	GNP4 III	Uncultured bacterium (*Rhizobium*/Agrobacteria group) EU429498.1	98	Soil samples from remote glaciated areas	*α-proteobacteria*
56	GCP8 III	Uncultured *Mycoplana* sp. EU705764.1	99	Phoenix Spacecraft Assembly	*α-proteobacteria*

57 GCN8III	Bacterium N3 EU567034.1	100	Antarctic ice	*α-proteobacteria*
58 ECP9 III	Antartic bacterium DQ906770.1	98	Antarctic seawater	*Flavobacteria*
58a ESP6 II	*Leeuwenhoekiella aequorea* AJ278780.1	98	Polar seas	*Flavobacteria*
59 GCP9 I	Uncultured *Klebesiella* sp. DQ279306.1	99	*Tuber magnatum*	*γ-proteobacteria*
60 ENN5 I	*Alcaligenaceae* AB461098.1	98	Soybean stem	*β-proteobacteria*
61 GCP9 I	*Alteromonadaceae* AM913910.1	96	*Saccharina latissima*	*γ-proteobacteria*
62 GCN2 III	Psychrobacter sp. AB302185.1	98	Antarctic krill	*γ-proteobacteria*
63 ESN1 II	Uncultured *Acinetobacter* sp. FJ192814.1	99	Phoenix spacecraft	*γ-proteobacteria*
63a GCN2 I	Acinetobacter johnsonii FJ263917.1	99	Blood culture	*γ-proteobacteria*
64 ESP1 II	*Paracoccus* sp. AY167832.1	98	Spacecraft assembly facilities	*α-proteobacteria*
64a ESP9 II	*Paracoccus* sp. AY167832.1	98	Spacecraft assembly facilities	*α-proteobacteria*
65 GSP7 III	*Aeromonas punctata* FJ168777.1	100	Aquatic environment	*γ-proteobacteria*
65a GCP5 III	*Aeromonas punctata* FJ168777.1	100	Aquatic environment	*γ-proteobacteria*
65b GCP6 III	*Aeromonas punctata* FJ168777.1	100	Aquatic environment	*γ-proteobacteria*
66 GNN3 III	*Pseudomonas stutzeri* DQ224384.1	100	Oil refinery	*γ-proteobacteria*
67 GCN5 III	*Aeromonas veronii* bv. *veronii* FJ233864.1	99	Aquaculture systems	*γ-proteobacteria*
68 GCP5 II	Gammaproteobacterium EF111226.1	98	Bogota River	*γ-proteobacteria*
69 GSP10 II	*Pseudomonas sabulinigri* EU143352.1	99	Black beach sand	*γ-proteobacteria*
69a GSP9 II	Pseudomonas sp AB021318.1	99	Marine	*γ-proteobacteria*
70 GSN8 II	*Pseudomonas veronii* FM162562.1	100	Polluted sediment	*γ-proteobacteria*
70a GSN6 I	Pseudomonas sp FJ006877.1	99	Freshwater	*γ-proteobacteria*
71 GCN6 I	*Psychrobacter faecallis* FJ613319.1	100	Deep sea sediment	*γ-proteobacteria*
72 GCP1 I	*Arthrobacter arilaitensis/arilaiti* EU240951.1	100	Reblochon cheese	*Actinobacteria*
73 ESP6 III	*Kocuria rosea* EU982904.1	100	Rhizosphere	*Actinobacteria*

Most of the isolates were closely related to bacteria obtained from aquatic environments (coastal, marine and estuarine waters) but also from a variety of cold or glaciated environments, including Antarctic seawater, polar seas, Arctic sea ice and Antarctic ice (table 6). Also several sequences affiliated with sequences from bacteria isolated from contaminated areas such as oil refineries, sewage sludge, arsenic-contaminate fields, uranium mining waste and activated sludge.

As stated before, isolates were affiliated with 5 Phyla (Figure 15): *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* (high G+C Gram-positive), *Firmicutes* (low G+C Gram-positive) and *Deinococci-Thermus*. Isolates belonging to the Phylum *Proteobacteria* were dominant at both layers.

The majority of the isolates belonged to the Class *γ-proteobacteria*, the rest of the isolates were assigned to *α-proteobacteria, β-proteobacteria, ε-proteobacteria, Actinobacteria, Flavobacteria, Sphingobacteria, Deinococci* and *Bacilli* (Figure 16). The class *γ-proteobacteria* was predominant in all samples. The high proportion of *γ-proteobacteria* in collections can be partly biased, since the isolation procedure may favour bacterial strains able to rapidly grow on nutrient-rich media and overall we used a selective media for *γ-proteobacteria* - GSP.

Beta and *ε-proteobacteria* and *Bacilli* were exclusively found on SML. Despite an apparently larger diversity on SML, the predominance of one single class (*γ-proteobacteria*) was more evident in this layer, in part due to the larger representativeness of *α-proteobacteria* in the deepest layer. Proportion of *Deinococci* and *Sphingobacteria* was very similar in both layers.

High diversity levels are common in estuarine samples from UW, with *α-proteobacteria*, *β-proteobacteria*, *γ-proteobacteria* and *Bacteriodetes* being widespread taxa (Crump et al., 1999, 2004; Henriques *et al*., 2004, 2006; Kisand and Wikner, 2003).

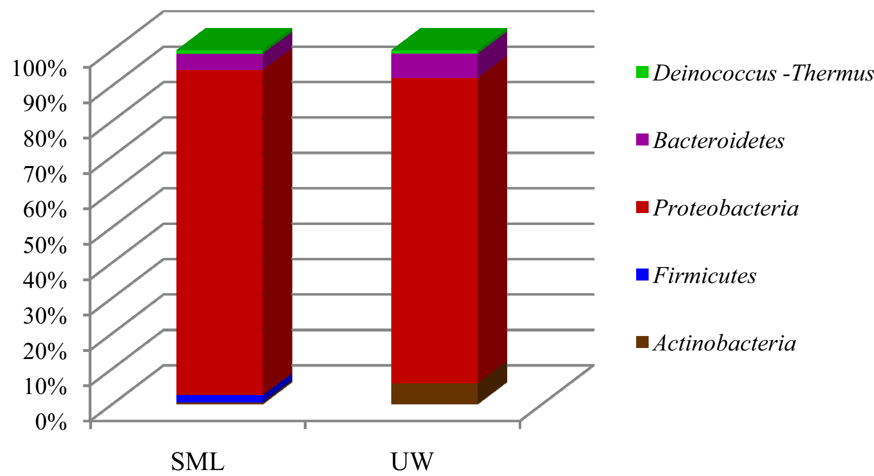

Figure 15: The proportions of taxonomic groups (Phyla) represented by the bacterial isolates from SML and UW samples.

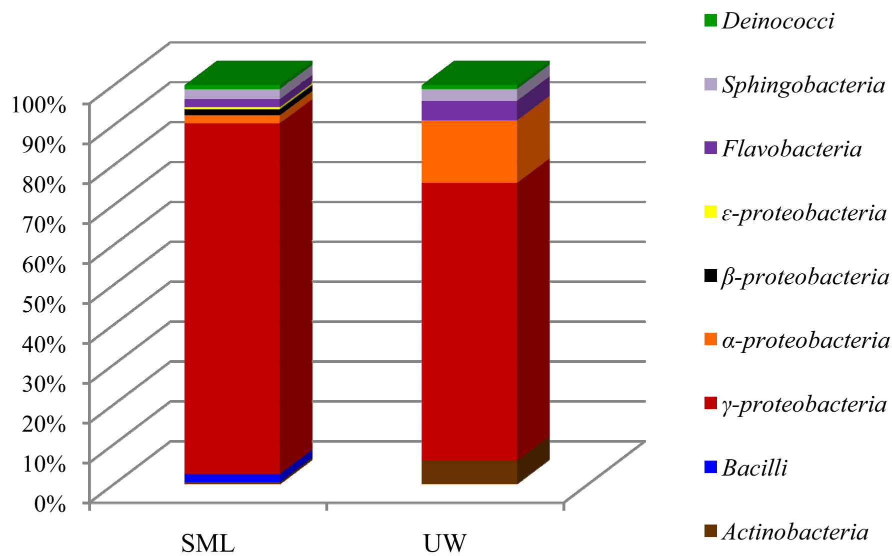

Figure 16: The proportions of taxonomic groups (Classes) represented by the bacterial isolates from SML and UW samples.

Taking into account the culture media, surprisingly, the higher diversity was recovered from GSP (Figure 17). This medium has been described as selective for *Pseudomonas sp.* and *Aeromonas sp.*; however, isolates that affiliated with 7 different classes were recovered from this medium.

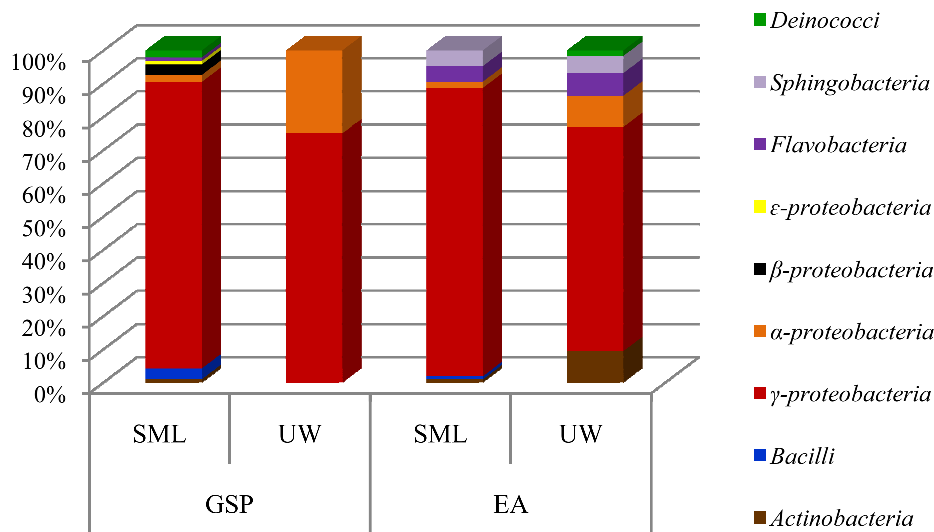

Figure 17: The proportions of taxonomic groups (Classes) represented by the bacterial isolates from SML and UW samples, isolated using GSP and EA agar plates.

Attending to the three performed campaigns (Figure 18), we can observe that *ε-proteobacteria* and *β-proteobacteria* as well as *Bacilli* were retrieved only from SML samples, in C3 for *ε-proteobacteria* while *β-proteobacteria* and *Bacilli* were only retrieved from C1 and C2. *Actinobacteria* only occurred once at SML, at one sampling site (CN) in C1, while it was always present in UW in all campaigns with some sampling sites exceptions (CN in C2 and CC in C3). The remaining classes were represented in both layers. *γ-proteobacteria* was always the most abundant class, probably a reflex from the followed methodology, namely the culture dependent approach and the chosen culture media.

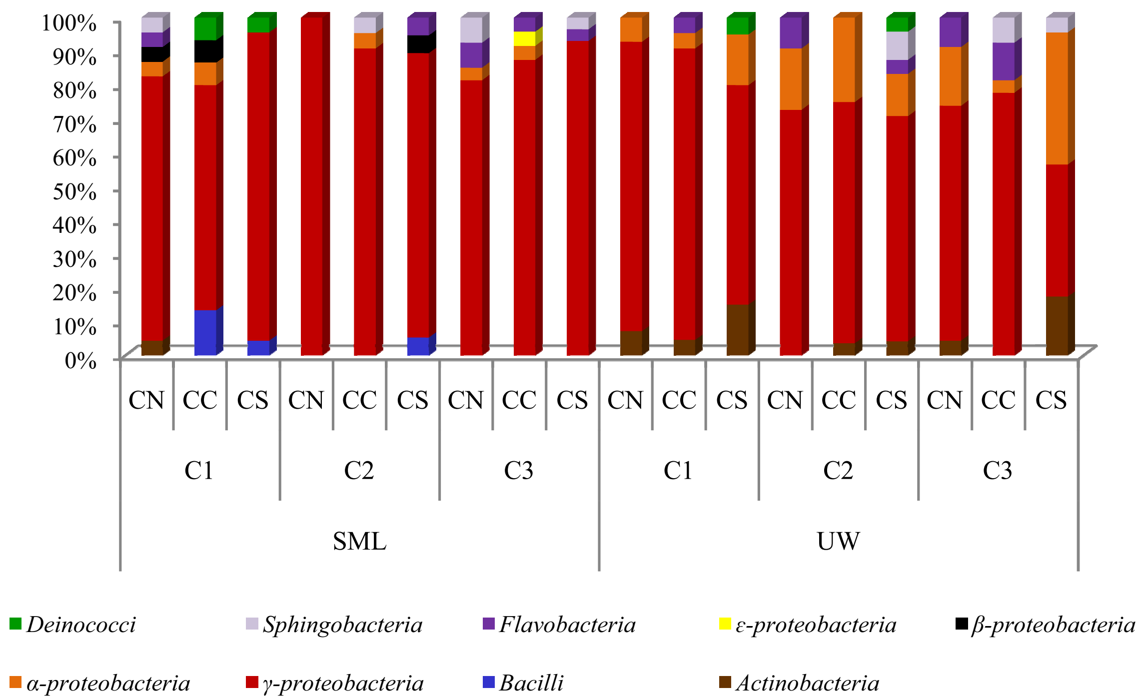

Figure 18: The proportions of taxonomic groups for the three campaigns (C1 to C3).

In terms of phylogenetic diversity no consistent differences were found concerning the diel cycle (figure 19). In fact, similar patterns were observed in day and night samples, for both SML and UW. *ε-proteobacteria* was the only class recovered exclusively from day samples. So, diel patterns of diversity were not observed.

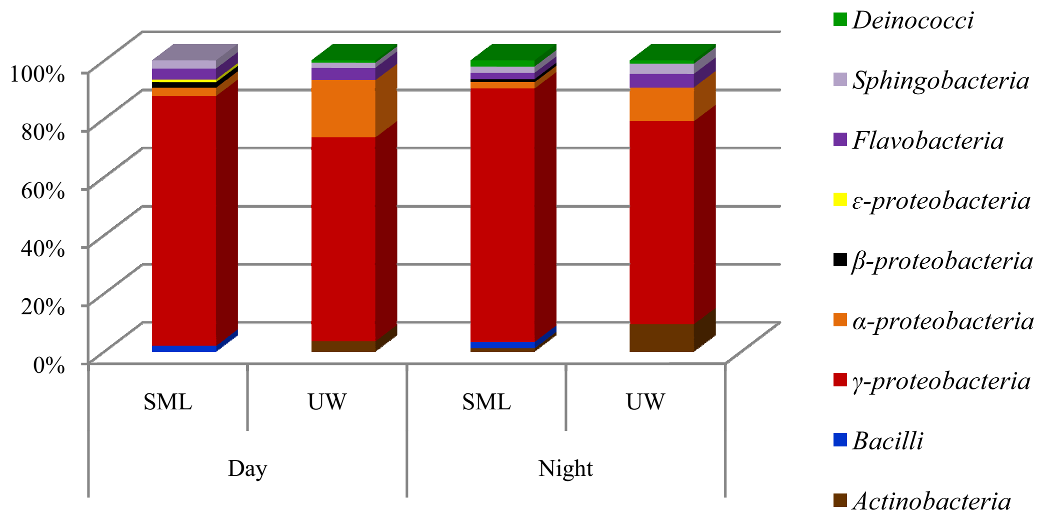

Figure 19: The proportions of taxonomic groups according to the sampling moment (day/night).

The spectrum of different genera was greatest within the Class *γ-proteobacteria* (11 genera), *α-proteobacteria* (5 genera) and *Flavobacteria* (4 genera). In figures 20-24 the proportions of genera within each class are shown. *ε-proteobacteria* and *Deinococci* are represented by only one genus each (*Arcobacter* and *Deinococcus*, respectively), *β-proteobacteria* sequences affiliated with uncultured representatives of genera *Comamonas* and *Alcaligenes* and the Class *Bacilli* was represented by two genera (*Bacillus* and *Aerococcus*, both from the same layer- SML); therefore, graphics for these classes are not presented here.

Class *α-proteobacteria*

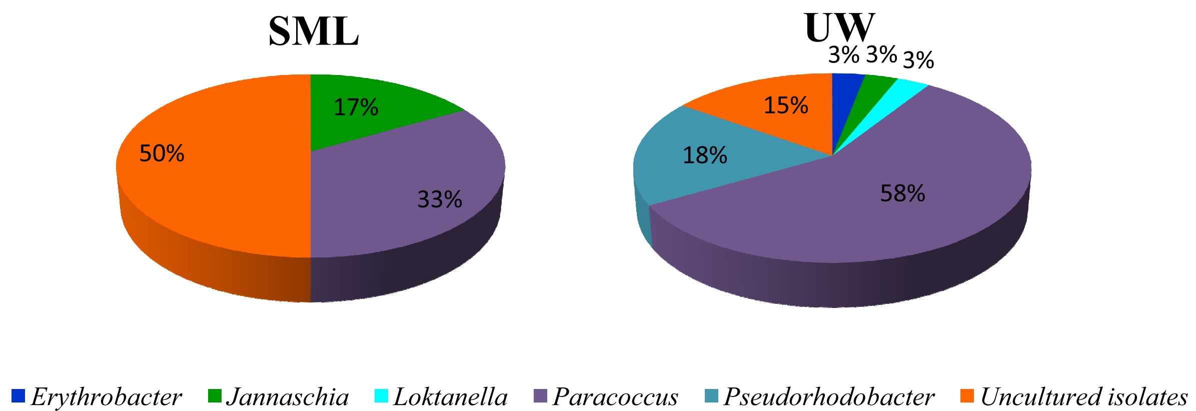

Figure 20: The Class *α-proteobacteria* proportions represented by the bacterial strains isolated from estuarine waters from both SML and UW samples. The isolated strains and their percentage per the taxonomic group is given together with the genus or class.

Alphaproteobacteria (Figure 20) is one of the classes displaying most pronounced differences between both layers. Three genera (*Loktanella*, *Pseudorhodobacter* and *Erythrobacter*) only appeared in UW. This class has a high percentage of isolates that affiliated with unculturable organisms. *Paracoccus* seems to be the more abundant genus in both layers (6 isolates from SML and 19 isolates from UW).

Indeed, *α-proteobacteria* are one of most widespread and most extensively studied groups within bacteria. However, for these bacteria as a whole very few or really no distinctive molecular or biochemical characteristics are known. They are abundant constituents of various terrestrial and marine environments (Giovannoni *et al*., 2005).

Class *Actinobacteria*

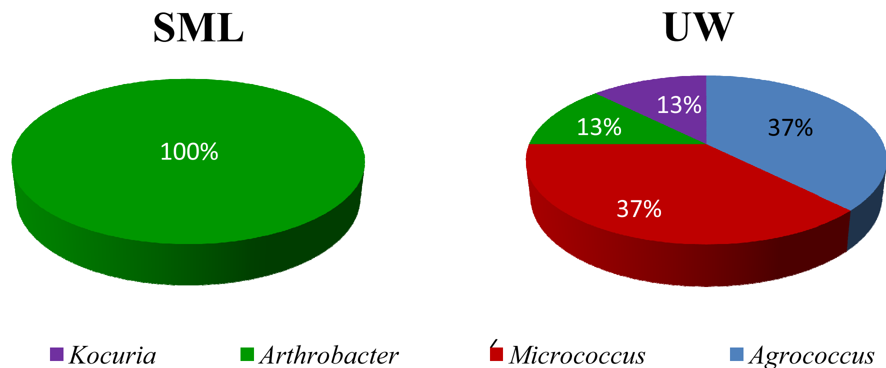

Figure 21 The Class *Actinobacteria* proportions represented by the bacterial strains isolated from estuarine waters from both SML and UW samples. The isolated strains and their percentage per the taxonomic group is given together with the genus or class.

Actinobacteria (Figure 21) were only represented in SML by the *Arthrobacter* genus. *Micrococcus* (3 isolates) and *Agrococcus* (3 isolates) were the predominant genera in UW.

Again, the extent of *Actinobacteria* diversity, abundance and biogeography remains unclear, because sampling is low and patchy, techniques are biased and *Actinobacteria* identification is not clearly defined (Ward and Bora, 2006).

Class *Flavobacteria*

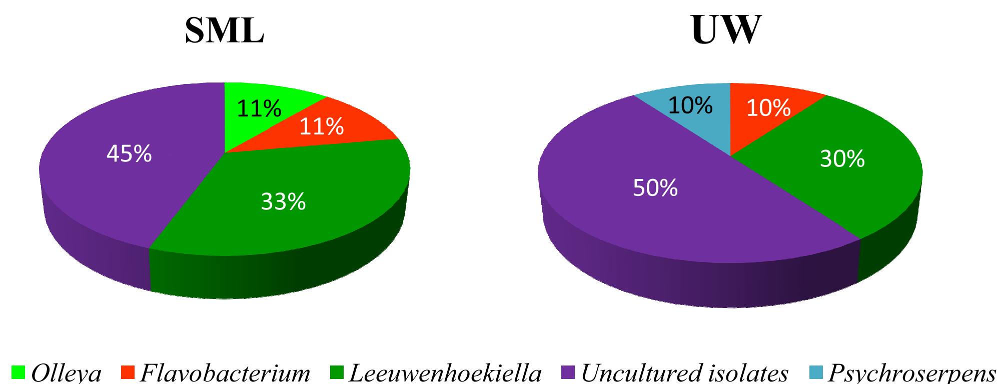

Figure 22: The Class *Flavobacteria* proportions represented by the bacterial strains isolated from estuarine waters from both SML and UW samples. The isolated strains and their percentage per the taxonomic group are given together with the genus or class.

The Class *Flavobacteria* (figure 22) has also a large proportion of uncultured isolates. *Leewenhoekiella* was the predominant genus in both layers (3 isolates from both).

Flavobacteria are generally commensal bacteria that live in soil and water and are opportunistic pathogens (Bernardet, 2005). *Flavobacteria* cluster is characterized mainly by aerobic species, particularly efficient at degrading complex polymers (Kirchman, 2002). Several studies reported high abundances during natural and induced phytoplankton blooms and subsequently bacteria belonging to this group are pointed out as primary colonizers of marine phytoplankton, suggesting a potential role as consumers of algae-derived metabolites (Simon *et al*., 1999; Riemann *et al*., 2000; O'Sullivan *et al*., 2004; Pinhassi *et al*., 2004; Grossart *et al*., 2005).

Class *Sphingobacteria*

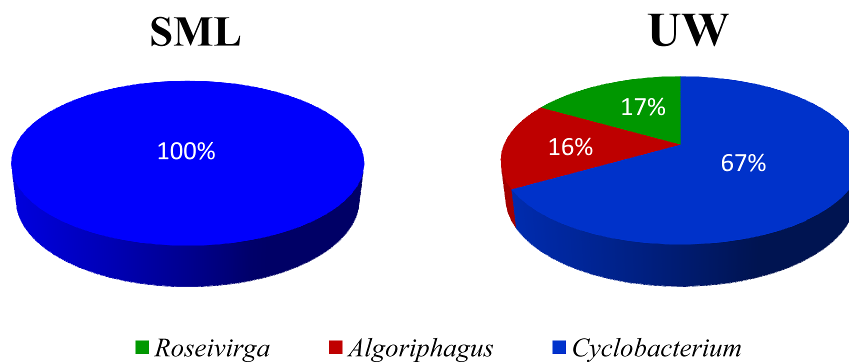

Figure 23: The Class *Sphingobacteria* proportions represented by the bacterial strains isolated from estuarine waters from both SML and UW samples. The isolated strains and their percentage per the taxonomic group is given together with the genus or class.

Sphingobacteria were only represented, in SML, by the *Cyclobacterium* genus. Indeed, it is also more abundant at UW, being this layer also enriched by bacteria belonging to genera *Roseivirga* and *Algoriophagus*.

An important feature of *Sphingobacteria* is the presence of high concentrations of sphingophospholipids as cellular lipid components which are ubiquitous components of

eukaryotic cell membranes although most bacteria do not possess them (Miyagawa *et al.*, 1978; Olsen and Jantzen, 2001).

Sphingobacteria are isolated from worldwide environments (Yabuuchi *et al.*, 1983; Shivaji et al., 1992) and include several clinical specimens (Holmes *et al.*, 1982). Also, bacteria belonging to the Class *Sphingobacteria* are capable of digesting various biopolymers, such as cellulose, chitin, pectin, starch and proteins (Reichenbach, 1991)

Class *γ-proteobacteria*

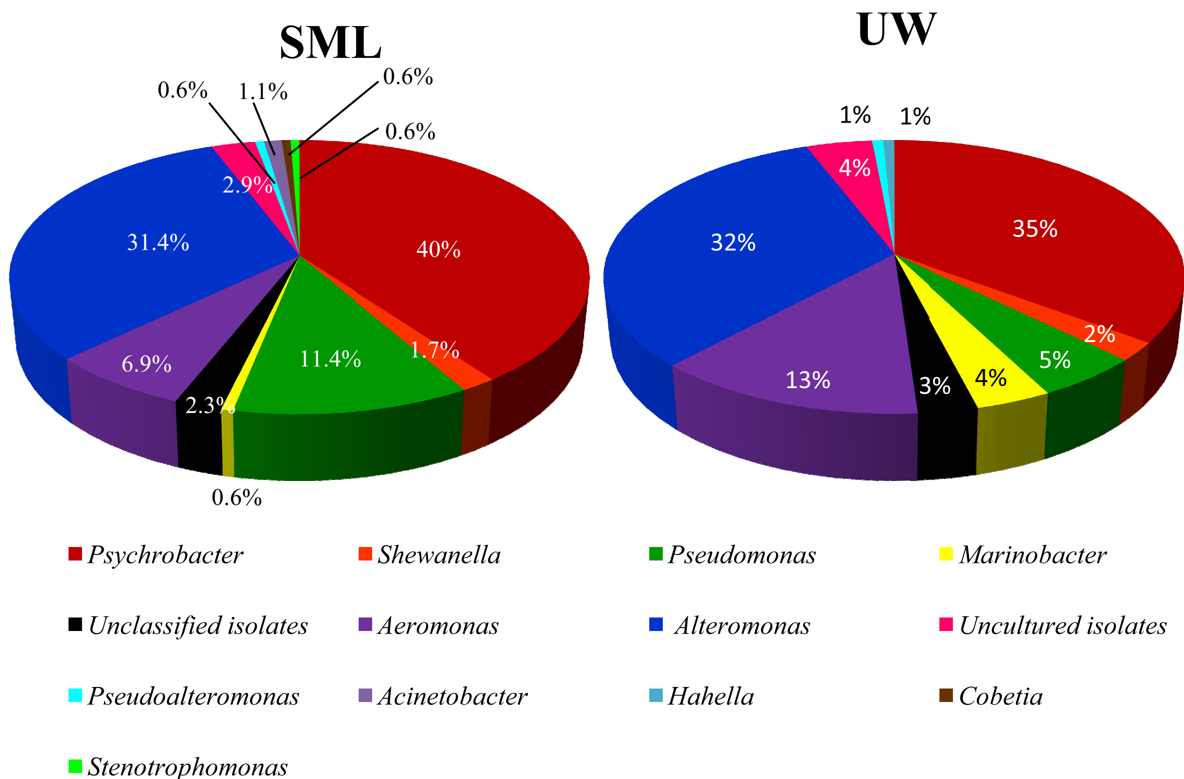

Figure 24: The Class *γ-proteobacteria* proportions represented by the bacterial strains isolated from estuarine waters from both SML and UW samples. The isolated strains and their percentage per the taxonomic group are given together with the genus or class.

γ-proteobacteria is the class represented by the larger number of different genera. *Psychrobacter*, followed by *Alteromonas*, were the most abundant genera in both layers.

Cobetia, *Stenotrophomonas* and *Acinectobacter* were only found in SML. By contrast, *Hahella* was exclusive from UW. However, we cannot infer conclusive remarks since only a few representatives from each of these genera were isolated.

γ-proteobacteria constitutes a large phylogenetic group of cosmopolitan species, generally well-represented in culture collections, ranging from clinical to environmentally important species (Bowman *et al*., 1997; Pinhassi *et al*., 1997; Suzuki *et al*., 1997; Eilers *et al*., 2000).

In summary, exploring the potential of the culture collection here established should be a matter of interest since there is an increase demand for organisms that naturally display features that can be used and optimized in order to solve environmental disorders, clinical-related problems and biotechnological issues.

4.5. Phylogenetic trees

The phylogenetic affiliation of the sequences obtained during this study was confirmed by constructing phylogenetic trees (Figure 25, 26 and 27).

PAUP_1

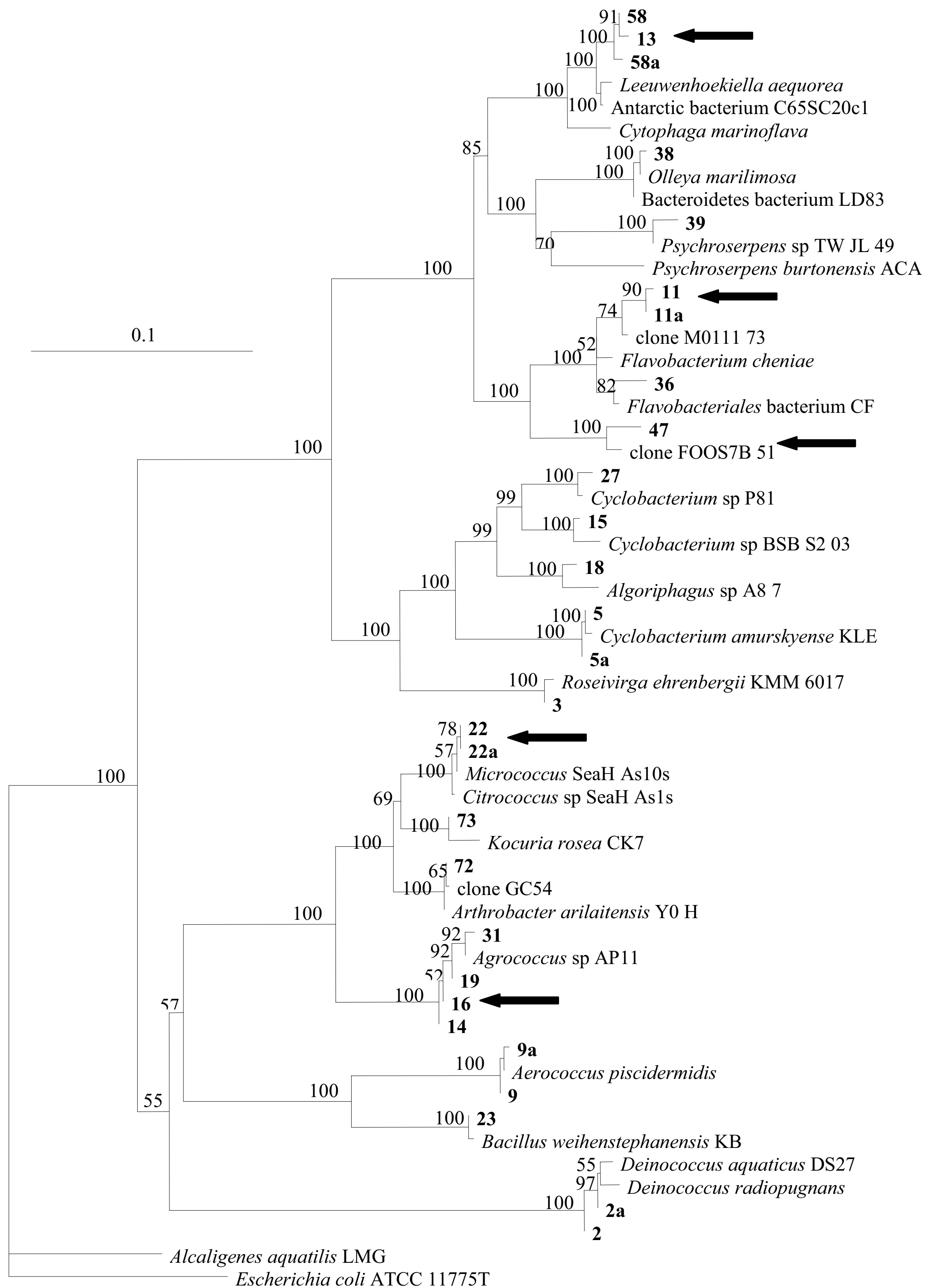

Figure 25: Phylogenetic tree based on 16S rDNA sequences (on average 900bp), showing relationships between all the recovered isolates from four different Phyla *Actinobacteria*, *Firmicutes*, *Deinococci-Thermus* and *Bacteroidetes*. Bootstrap values (1000 replicates) above 50% are shown at nodes.

PAUP_1

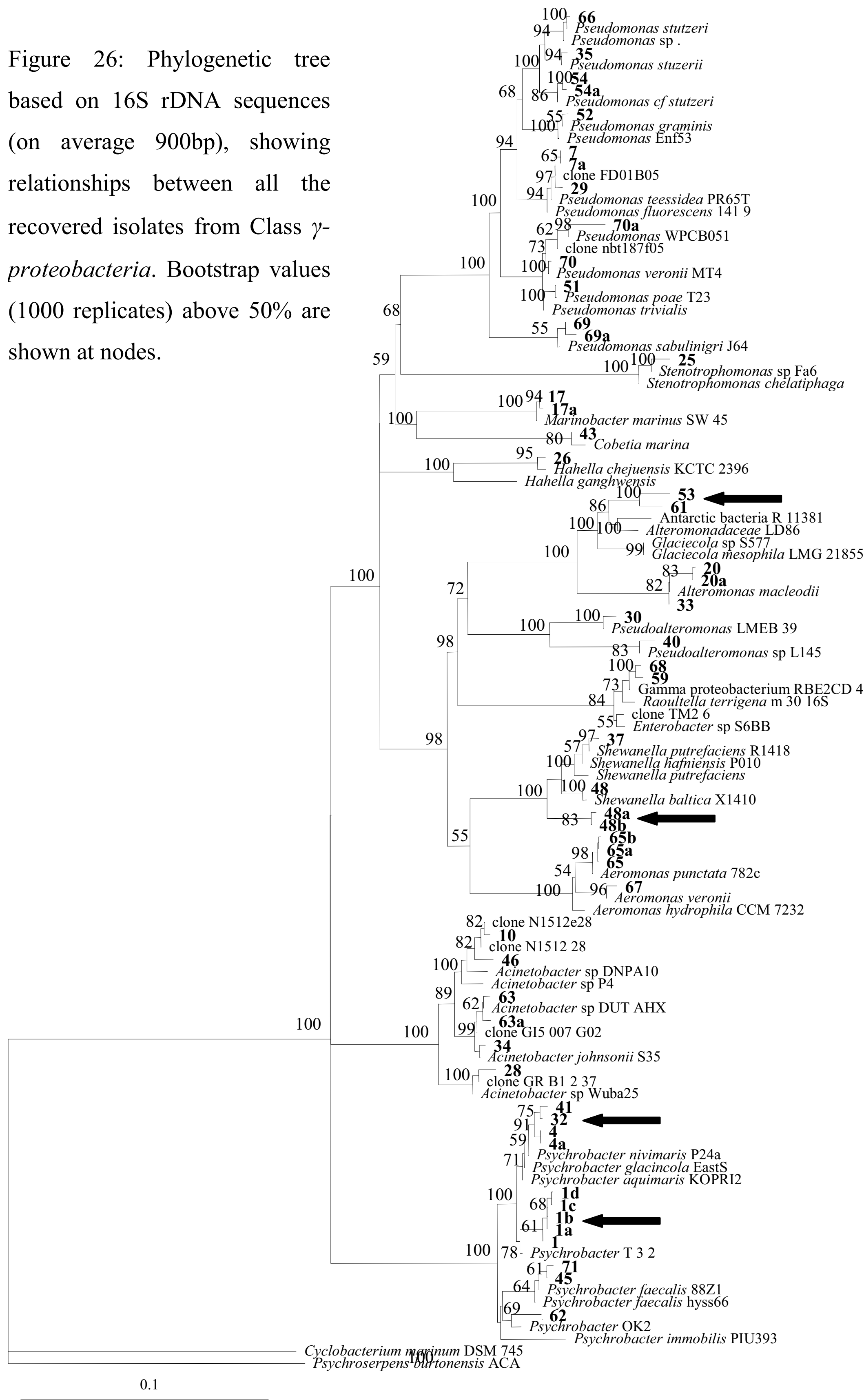

Figure 26: Phylogenetic tree based on 16S rDNA sequences (on average 900bp), showing relationships between all the recovered isolates from Class *γ-proteobacteria*. Bootstrap values (1000 replicates) above 50% are shown at nodes.

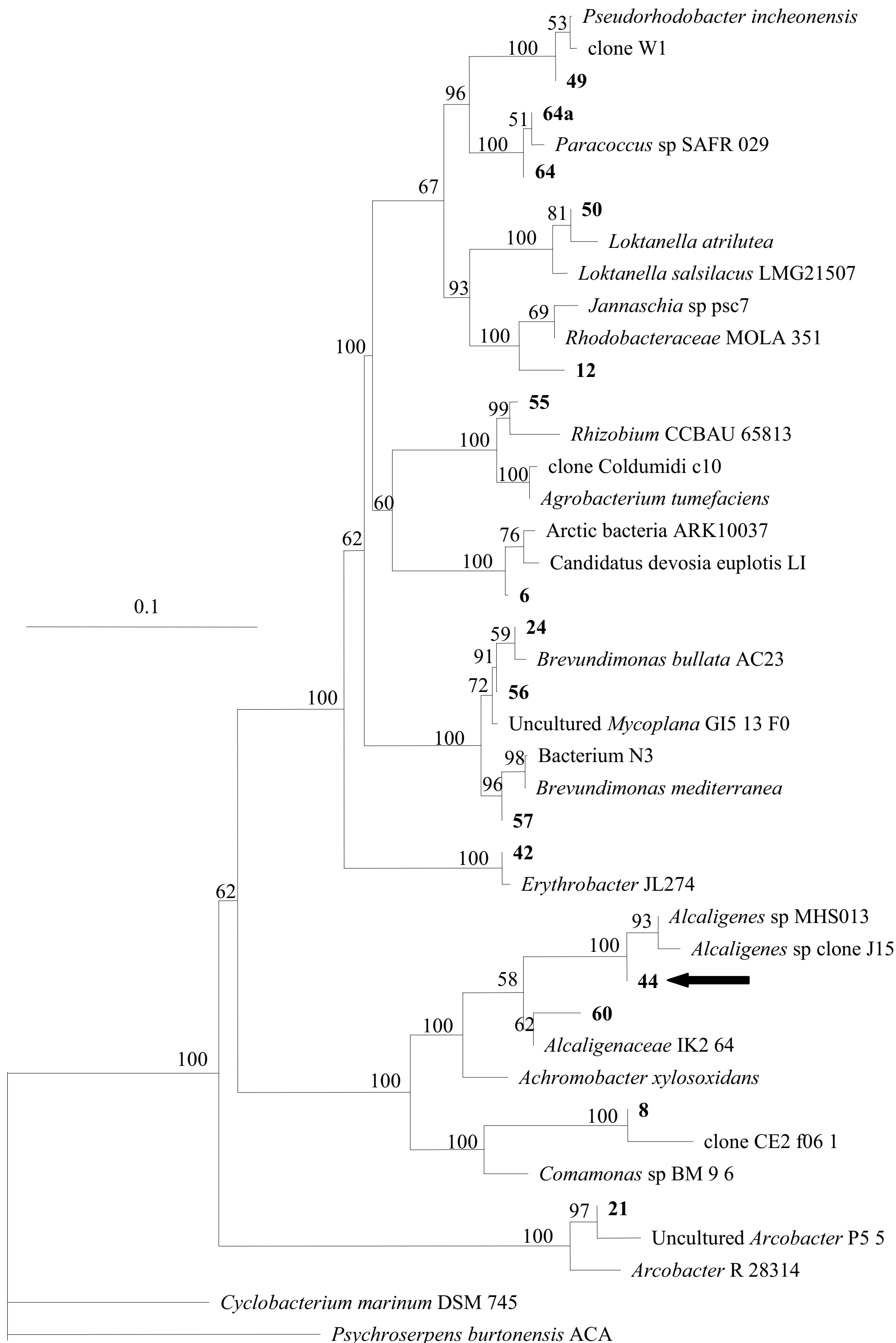

Figure 27: Phylogenetic tree based on 16S rDNA sequences (on average 900bp), showing relationships between all the recovered isolates from *α-proteobacteria*, *β-proteobacteria* and *ε-proteobacteria*. Bootstrap values (1000 replicates) above 50% are shown at nodes.

The phylogenetic analysis allowed us to confirm the affiliation of the retrieved sequences. In some cases sequences retrieved during this study grouped in distinct clusters only distantly related with clusters including previously described species (some examples are indicated with arrows within phylogenetic trees). This result suggests that some of the sequences can probably represent yet undescribed species. However to confirm this hypothesis further studies are needed.

Besides the well known and reported bias inherent to cultivation and all the constraints arising from this methodology, culture still is a fundamental step for the study of microorganisms. Two decades after a microbial ecology based upon cultivation-independent methods and when prokaryote cultivation was seen as a failed technology, the need for new culture techniques that permit the isolation of major uncultivated clades in order to fully understand it have revived the cultivation effort (Rappe *et al*., 2002; Stevenson *et al*., 2004)

Indeed, bringing representative environmental clades into culture is essential to link function to community structure and to provide reference scaffolds for metagenome assembly (DeLong and Karl, 2005; Giovannon and Stingl, 2005).

5. Conclusion

The main aims of the present study were to characterize differences between the SML and UW in terms of abundance and diversity of culturable heterotrophic bacteria and to establish a culture collection of strains obtained from both layers. From the obtained results several conclusions could be drawn:

1) Within the estuary Ria de Aveiro higher abundances of culturable bacteria within the SML when compared to UW were obtained, independently of the sampling site, sampling date and culture media.

2) Diel cycles of abundance were detected: SML bacteria were remarkably more abundant during day than night while for UW bacteria significant differences were not observed.

3) In terms of phylogenetic diversity significant differences between layers were also observed, suggesting that SML and UW are in fact inhabited by distinct heterotrophic communities.

4) The extent of abundance and diversity differences between both layers was dependent of the sampling site and the sampling moment. However further studies are needed to elucidate the relevance of seasonal and spatial factors in determining differences between SML and UW bacterial communities.

5) A culture collection of bacterial isolates comprising a high diversity (isolates belonging to 5 phyla and 9 different classes) was established. Some of the retrieved sequences can probably represent yet undescribed taxa.

6. Future perspectives

Further studies are needed to answer a number of questions that can be drawn from the analysis of the results obtained during this study. Probably some of these issues should be assessed by combining cultivation efforts along with independent-cultivation approaches.

Specifically the diel patterns of abundance and diversity of SML and UW communities deserve further attention. Differences observed during the present study should be confirmed and characterized and the correlation between these patterns and the UV radiation regimens should be determined.

On the other hand, the preliminary results here obtained concerning antibiotic resistance within both layers point also to an interesting theme of discussion. The relevance of this thematic justifies further studies to understand why SML bacteria are more susceptible to antibiotics that UW bacteria.

Finally the culture collection here established constitutes a resource that should, and certainly will, be the object of a number of studies aiming to explore for example its biotechnological potential, the presence of potentially new species, the presence of antibiotic resistance determinants and mobile genetic elements and the expression of extracellular enzymes.

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Annexes

Annex 1

Table7: Levels of salinity and temperature (ºC) registered in the five campaigns. (Mean fallout, Vmax= maximum value and Vmin= minimum value).

		Salinity			Temperature (ºC)		
		Mean	**Vmax**	**Vmin**	**Mean**	**Vmax**	**Vmin**
C1	CN	4.25	4.6	3.9	16.95	17.9	16
	CC	1.85	1.9	1.8	15.95	16	15.9
	CS	4.95	6.1	3.8	16	16	16
C2	CN	16.5	16.5	16.5	19.15	19.7	18.6
	CC	14.05	14.7	13.4	18.7	18.9	18.5
	CS	22.95	24.6	21.3	19.25	19.5	19
C3	CN	23.5	23.5	23.5	20.4	20.6	20.2
	CC	18.7	18.7	18.7	21.5	21.7	21.3
	CS	29.25	29.3	29.2	20.25	20.8	19.7
C4	CN	27.5	27.5	27.5	20.05	20.5	19.6
	CC	29.2	29.5	28.9	20.35	20.9	19.8
	CS	33.2	33.3	33.1	18.8	18.8	18.8
C5	CN	27.85	27.9	27.8	19.6	19.8	19.4
	CC	29.9	30.1	29.7	20.6	21.6	19.6
	CS	33.1	33.1	33.1	19.1	19.2	19

Annex 2

Profile	Total number of isolates	Number of isolates from SML samples	Number of isolates from UW	Number of sequenced representatives	Sequenced isolates (Code)
1	**102**	61	41	5	GNN1III GSN5 III ESP9 I ENP6 II GNN8 III
2	**4**	2	2	2	ESP7II GSN9 I
3	**1**	0	1	1	ESP3 II
4	**7**	3	4	2	GSP8 II GSP2 II
5	**6**	3	3	2	ESP4 II ECP6 III
6	**1**	1	0	1	ECN8 I
7	**3**	2	1	2	GSN5 II GCN9 II
8	**1**	1	0	1	GCN4 I
9	**3**	3	0	2	GCN9 I GSN1 II
10	**1**	0	1	1	GCP8 II
11	**4**	1	3	2	ENP10 III ECP7 III
12	**2**	1	1	1	ENP2 III
13	**1**	0	1	1	ENP4 III
14	**5**	0	5	1	ENP5 III
15	**2**	1	1	1	ECP7 III
16	**1**	0	1	1	ESP8 II
17	**6**	1	5	2	GNP6 II GCP2 II
18	**1**	0	1	1	ECP10 I
19	**1**	0	1	1	ESP5 I
20	**98**	53	45	2	ESN8 III ESP5 III

21	**1**	1	0	1	GCN6III
22	**2**	0	2	2	ESP5 III ESP6 II
23	**1**	1	0	1	ESN7 I
24	**1**	1	0	1	GCNP1II
25	**1**	1	0	1	GSN4 I
26	**1**	0	1	1	ECP1 II
27	**1**	1	0	1	ENN8 I
28	**1**	0	1	1	ECP6 I
29	**1**	1	0	1	ECN7 I
30	**1**	1	0	1	GSN8 III
31	**1**	0	1	1	ESP5 I
32	**1**	0	1	1	GSP8 II
33	**2**	2	0	1	ENN7 III
34	**1**	0	1	1	GCP10 III
35	**1**	1	0	1	GSN6 III
36	**1**	0	1	1	ENP10 III
37	**1**	0	1	1	ECP9 III
38	**1**	1	0	1	ESN4 II
39	**1**	0	1	1	ENP5 II
40	**1**	0	1	1	ESP3 I
41	**1**	0	1	1	GSP3 II
42	**1**	0	1	1	ENP6 III
43	**1**	1	0	1	ENN3 II
44	**1**	1	0	1	GSN9 II
45	**1**	1	0	1	ENN10 II
46	**1**	0	1	1	GCP3 III
47	**1**	1	0	1	GNN5III
48	**5**	3	2	3	GNN5 I ECP10 III ECP3 III
49	**6**	0	6	1	GSP3III
50	**1**	0	1	1	ENP8 II
51	**10**	9	1	1	GCN9 III
52	**1**	1	0	1	GSN1 II
53	**7**	4	3	1	ESP1 II

54	**3**	3	0	2	GSN2 III GNN9 III
55	**1**	0	1	1	GNP4 III
56	**4**	0	4	1	GCP8 III
57	**1**	1	0	1	GCN8 III
58	**5**	3	2	2	ECP9 III ESP6 II
59	**1**	0	1	1	GCP9 I
60	**1**	1	0	1	ENN5 I
61	**1**	0	1	1	GCP9 I
62	**4**	3	1	1	GCN2 III
63	**5**	4	1	2	ESN1 II GCN2 I
64	**22**	2	20	2	ESP1II ESP9 II
65	**29**	11	18	3	GSP7 III GCP5 III GCP6 III
66	**2**	2	0	1	GNN3III
67	**1**	1	0	1	GCN5 III
68	**1**	0	1	1	GCP5 II
69	**4**	0	4	2	GSP10 II GSP9 II
70	**4**	3	1	2	GSN8 II GSN6 I
71	**2**	1	1	1	GCN6 I
72	**2**	1	1	1	GCP1 I
73	**1**	0	1	1	ESP6 III

Table 8: Obtained ARDRA profiles and total number of isolates displaying each profile and the number of those recovered from SML samples or from UW samples; also the number of sequenced isolates for each profile is presented.

UW exclusive (28)

SML exclusive (21)

Annex 3

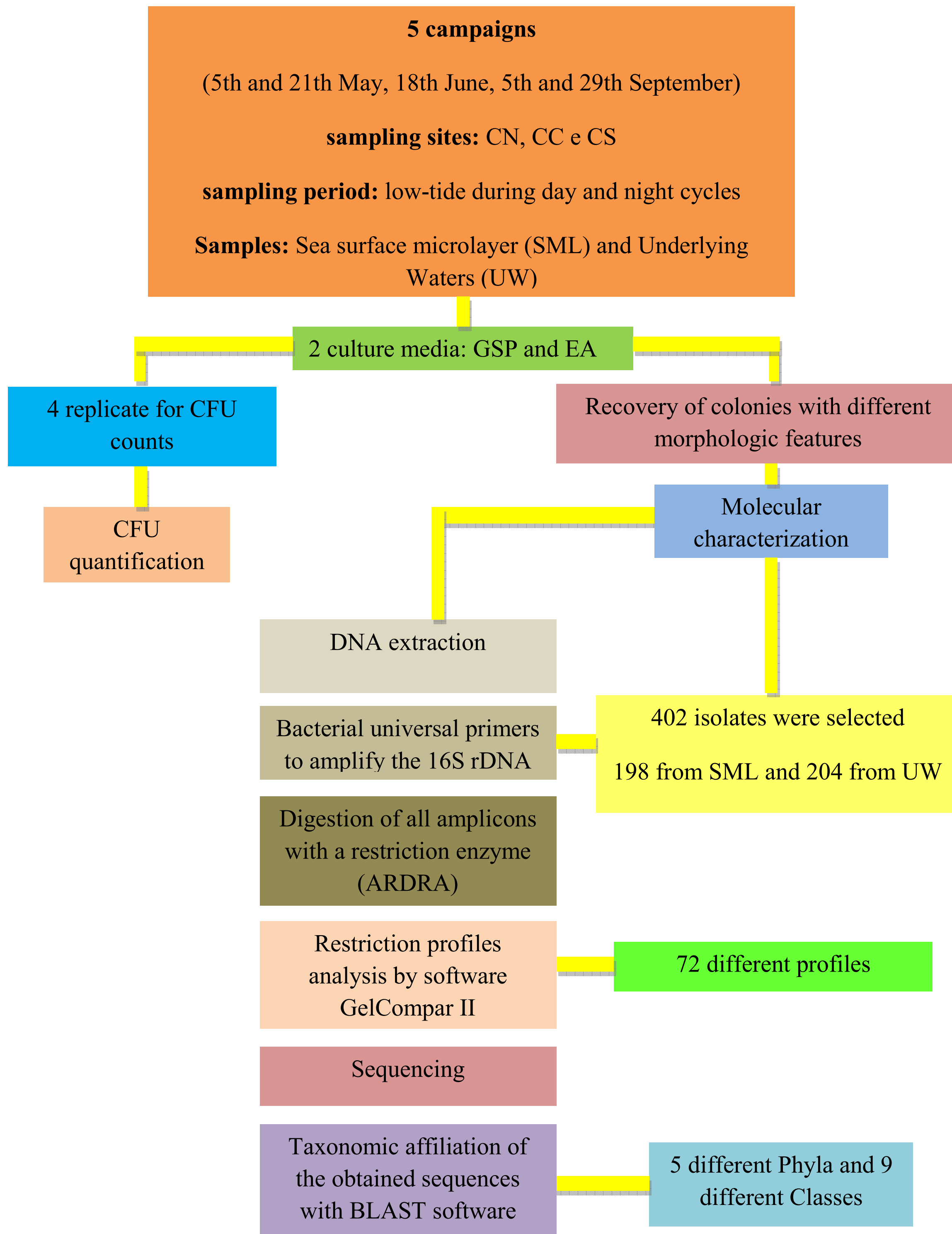

Figure 28: Work project concerning the methodology applied (culture dependent methods), the sampling planning and the results obtained – Organigramm.