Diversity and biotechnological potential of epiphytic bacteria of macroalgae

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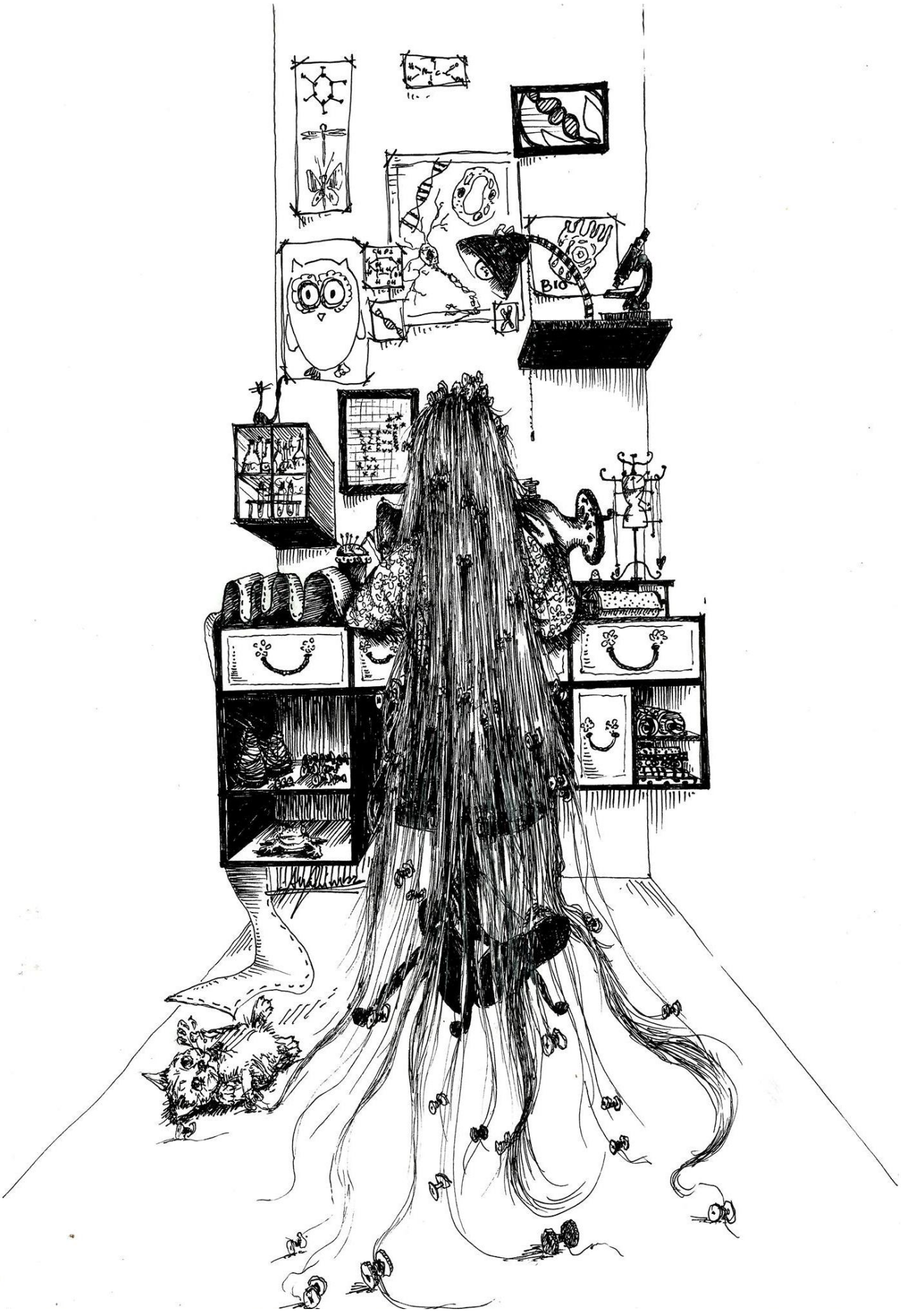
BIO

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Resumo

Os oceanos são um ecossistema com uma elevada diversidade de espécies, contendo milhões de microrganismos, muitos deles ainda por descobrir. Com os avanços tecnológicos, novas espécies microbianas têm sido descritas e demonstraram ser de extrema relevância para o ambiente e qualidade de vida humana. As bactérias são os principais intervenientes nos processos ecológicos e biogeoquímicos que ocorrem no ecossistema marinho. São encontradas na superficie de vários macroorganismos formando complexas associações denominadas biofilmes, com importância ecológica.

As superfícies das macroalgas são comummente colonizadas por uma diversa comunidade bacteriana que tende a ser específica e constante em diferentes espécies de macroalgas. As complexas interações entre as macroalgas e as bactérias revelam-se cruciais para o desenvolvimento de ambos os organismos. Novas espécies e géneros de bactérias têm sido encontradas no biofilme da superficie das macroalgas por métodos dependentes e independentes de cultivo.

Para além disso, as bactérias comunicam entre si e com o hospedeiro através de interações complexas mediadas pela produção de pequenas moleculas que podem ter potencial bioactivo. O conhecimento do funcionamento destes processos tem permitido descobrir compostos bioactivos com potencial biotecnológico.

Neste sentido, o objetivo principal deste estudo é analisar a diversidade bacteriana que se encontra no biofilme de três diferentes espécies de macroalgas: *Ulva* sp., *Porphyra dioica* e *Sargassum muticum* através de diferentes métodos de cultivo e moleculares. A observação da superficie das macroalgas por microscopia óptica e eletrónica de varrimento permitiu visualizar esta diversidade. Através do isolamento de bactérias em culturas puras, através de amostras das macroalgas amostradas no Outono, obtiveram-se 245 isolados (41% da *Ulva* sp., 25 % da *P. dioica* e 34 % do *S. muticum*). Até ao momento, apenas 86 culturas foram identificadas com base no gene do rRNA 16S, tendo-se obtido bactérias que pertencem ao filo das *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Firmicutes* e *Actinobacteria*. *Vibrio* foi o género mais abundante nos isolamentos.

A partir de macroalgas colhidas no Outono e Primavera, foram obtidos isolados de planctomycetes os quais são filogenéticamente afiliados a *Rhodopirellula baltica*.

Ambos os métodos de pirosequenciação (Sequenciação de nova geração) e de Eletroforese em Gel de Gradiente de Desnaturação (DGGE) permitiram verificar que

as macroalgas e a água do mar envolvente não apresentaram diferenças significativas entre as suas comunidades bacterias, e que o mesmo ocorreu em termos sazonais.

O estudo do potencial biotecnológico das bactérias isoladas foi realizado pela análise dos genes que codificam para policetídeo-sintases (PKS) e para sintetases de peptídeos não ribossomais. A amplificação destes genes ocorreu em cerca de 30% dos isolados mostrando um nivel apreciável de potencial bioactivo.

Com o objectivo de identificar bactérias com genes responsáveis pela comunicação interbacteriana, o gene luxS foi estudado. Apesar de várias tentativas, até ao momento não foi conseguida a sua amplificação inviabilizando o estudo do "*quorum-sensing*".

Na generalidade, os resultados obtidos demonstraram que esta diversidade é a que está normalmente associada à comunidade epifítica das macroalgas.

Palavras-chave: Macroalgas, biofilmes, bactérias, diversidade, potencial bioactivo, DGGE fingerprinting, pirosequenciação, quorum sensing

Abstract

Oceans are ecosystems that encompass a high diversity of species and include millions of microorganisms, many of them still undiscovered. Through the technological advances, novel microbial species have been discovered and show to be extremely important for environment and human well-being.

Bacteria are the main key players involved in ecological and biogeochemical processes on the marine ecosystem and appear in complex interactions called biofilms at the surface of macroorganism. Macroalgae surfaces are normaly colonized by a diversity of bacterial communities that shows to be stable and specific for each macroalgae species. Furthermore, the interactions between macroalgae and bacteria are essential in the development of both organisms. Novel bacteria genera and species have been found associated to the biofilm of macroalagae surface by independent culture-dependent and culture-independent techniques. Moreover, bacteria communicate with others and the host through complex interactions that are mediated by the production of small molecules usually with bioactive potential. The knowledge of the functioning of these processes allowed the discovery of the secondary metabolites with biotechnological potential.

Thereafter, the main goal of this study was to analyze the bacterial diversity present in the biofilm associated with three different species of macroalgae: *Ulva* sp, *Porphyra dioica* and *Sargassum muticum*, and compare this diversity through different culture and molecular methods. Optical and scanning electron microscopy allowed the observation of this diversity.

Through cultivation of pure bacterial cultures obtained from the macroalgae in Autumm, 245 isolates were obtained (41% from *Ulva* sp., 25 % from *P. dioica* and 34 % from *S. muticum*). Until now, 86 isolates were identify based on the 16S rRNA gene and *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Firmicutes* and *Actinobacteria* were obtained. *Vibrio* was the most abundant genus in the isolations. From macroalgae sampled in Autumn and Spring, planctomycetes isolates phylogeneticaly affiliated to the *R. baltica* were obtained.

Both pyrosequencing and Denaturing Gradient Gel Electrophoresis (DGGE) methods showed that no significant differences existed among the bacterial communities from macroalgae and surrounding seawater and that the same result was obtained in terms of sazonality.

The study of the biotechnological potential of the isolated bacteria was done based on the analysis of the Polyketide synthases (PKS) and Nonribosomal peptide

sinthethases (NRPS) genes. The amplification of these genes ocorred in about 30% of the isolates, showing a considerable level of bioactive potential.

For the identification of bacteria that produce genes responsible for the bacterial communication, the presence of luxS gene was study. However, after several attempts, no amplification was obtained until now, making impossible the study of the quorum-sensing.

In general, our results showed that this diversity is the one usually associated to the epiphytic community of macroalgae.

Keywords: Macroalgae, biofilm, bacteria, diversity, bioactive potential, DGGE fingerprinting, pyrosequencing, quorum sensing.

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Abbreviations

®	registered trademark
™	trademark symbol
ºC	degree Celsius
µL	microlitre
µM	micromolar
Mm	micrometre
%	percent sign
16S rRNA	16S ribosomal ribonucleic Acid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	base-pair
CFU	Colony-forming unit
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
e.g	*exempli gratia*
EPS	Extracellular polymeric Substance
G	gram
M	Molar
MA	Marine agar medium
MF	Medium F
mL	milliliter
Mg	milligram
$MgCl_2$	Magnesium Chloride
mM	milliMolar
Min	minutes
mS/cm	milliSiemens per centimetre
Ng	nanogram
NGS	Next-generation sequencing
NRPS	Nonribosomal peptide sinthethases
OM	Optical microscopy
OTU	Operational Taxonomic Unit
PAST	Paleontological statistics software package
PCR	Polimerase chain reaction

pH	Potential of Hidrogen
PKS	Polyketide synthases
Ppi	Inorganic pyrophosphate
Ppt	Parts per thousand
QS	Quorum sensing
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Seconds
SEM	Scanning electron microscopy
sp.	Specie
ST	Starch marine medium
TAE	Tris-acetate
UV	Ultraviolet

1. Introduction

1.1. Marine microorganisms – Overview

The world oceans are the largest ecosystem on earth (Rumney, 1968). It covers over 70% of the earth's surface and contains a rich diversity of microorganisms estimated in several millions different species (Whitman *et al*., 1998). The total number of prokaryotic cells in the oceans is 10^{29} (Whitman *et al*., 1998) with 10^{6} bacterial taxa suggested (Pedrós-Alió, 2006). Per mL of seawater it is possible to find a range of millions of viruses and bacteria, thousands of fungi and microalgae, and hundreds of microscopic larvae and spores (Harder, 2009). In recent years the interest in understanding the function of marine ecosystems has been accelerated because of its increasing impact on human life. However, and in general, marine microbes have not been studied as extensively as their terrestrial counterparts (National Research Council (US) Committee on Molecular Marine Biology, 1994). The impossibility to observe in great detail microorganisms by direct methods allied to difficulties in sampling some marine inaccessible areas delayed the increasing knowledge of marine microbial species (DeLong and Pace., 2001; Brandt *et al*., 2014). In spite of their importance, knowledge of marine microbial diversity is insufficient in terms of quantity and quality of the microorganisms present in the oceans and their ecological interactions and functions. Only in the late 19th century with the development of pure-culture techniques it was possible to enlarge our knowledge on microbial species and their characteristics. However, this approach only provides limited information about the organisms that can grow under determined conditions restricting the knowledge of microbial diversity (Pace, 1997). It is estimated that only 1% of marine bacteria can be isolated by cultivation methods (Amann *et al.,* 1995). The development and application of novel and powerful molecular tools allowed increasing greatly the analysis of microbial diversity and of the community dynamics in their environment. In the early 90's the analyses of ribosomal RNA (rRNA) sequences permitted to organize all living organisms into three major Domains: *Bacteria* and *Archaea* (both forming the "Prokaryotes") and *Eukarya* (Woese *et al.,* 1990). As the molecular markers rRNA genes have a highly conserved nature that anneal with "universal" PCR primers, they allowed the demonstration of the evolutionary relationships between all organisms and the reorganization of the phylogenetic tree (Woese *et al.,* 1990; Pace, 1997). The 16S rRNA gene has been used for the study of bacterial phylogeny and taxonomy (*e.g.* Fox *et al.,* 1977; Woese and Fox, 1977; Weisburg *et al*., 1991; Coenye and Vandamme, 2003; Woo *et al*., 2008). This genetic marker is considered a universal bacterial

identification tool as it is present in all bacteria, often existing as a multigene family, or operon; its function do not change over time (Woese,1987) and the size of the gene (approximately 1500 bp) is sufficiently large for informatic purposes as it contains statistically relevant sequence information (Patel, 2001). The 16S rRNA molecule possesses approximately 50 functional domains. The number of domains is important because the introduction of selected changes in one domain does not greatly affect sequences in other domains and these changes have lower impact on phylogenetic relationships (Woese, 1987). The discovery of 16S rRNA gene became an important milestone for detection, classification and reclassification of numerous bacterial species and genera and also for the discovery of uncultivable bacteria (Janda and Abbott, 2007; Woo *et al*., 2008).

In the sea world, prokaryotes encompass the majority of the genetic diversity (Glockner *et al*., 2012) and represent the second most abundant group after the viruses (Breitbart, 2012). The ubiquity of *Bacteria* and *Archaea* is favoured by their widespread dispersal capacity, metabolic flexibility and versatility combined with the fact that these organisms have the capacity to resist to extreme and antagonistic conditions (Schlegel and Jannasch, 2006). They play an essential role in ecology and biochemistry cycles, energy flow and nutrition (Arrigo, 2005; Thakur *et al*., 2008). For this reason, during evolutionary times these organisms have been playing an important role in the chemistry of the oceans and the atmosphere (Redfield, 1958) and are responsible for 50% of the global primary production occurring in the marine environment (Odeyemi, 2013). In bacteria, prochlorophytes and cyanobacteria are the main organisms that contribute to phytoplankton biomass and are responsible for energy flow and microbial food webs cycling in oligotrophic oceanic ecosystems (Sherr and Sherr, 1991; 1994). In the oceans, bacteria had to adapt to various, sometimes harsh, environments which implicated the development of diverse metabolisms and surviving strategies. Additionally, bacteria have the capacity to interact in different ways with marine macroorganisms, like macroalgae, sponges, anemones and other invertebrate animals (Bewley and Faulkner, 1998; Goecke *et al.,* 2010; Barott *et al.,* 2011; Graça *et al*., 2015), and are capable of colonizing their surface forming biofilms (Weinberger, 2007). Furthermore, surviving strategies led bacteria to produce bioactive compounds which may be used for many applications (Proksch *et al*., 2002; Graça *et al*., 2015). For example, actinomycetes have been an important bacterial group in the research of new products with antibiotic and anticancer potential (Goodfellow and Haynes, 1984; Jensen *et al*., 1991; Kim *et al*., 2005; Kim *et al*., 2006) and also in the recycling of organic matter (Srinivasan *et al*., 1991). So, bacteria are, thus, environmentally relevant as key players in all major biogeochemical cycles, energy fluxes, processes in

marine ecosystems and also to improve the human well-being by their biotechnological potential.

1.2. Marine bacterial diversity

As a liter of seawater contains in general 10^9 bacterial cells (Curtis *et al*., 2002), it is evident that the marine environment is the shelter of an enormous bacterial diversity (Giovannoni and Stingl, 2005; Zinger *et al*., 2011). This varies due to changes in water temperature, salinity, nutrients and other physicochemical parameters (Alavandi, 1990) and is also controlled by biological interactions. The interest to determine bacterial marine diversity has been important to understand communities' structure, distribution and function but also to identify their ecological importance for the support of other organisms and ecosystems. The marine environment encompasses about 20 recognized culturable and well-studied major phyla of *Bacteria* (Ciccarelli *et al*., 2006). Some of these divisions are represented in Table 1.

Table 1 – Some bacterial species found in marine ecosystems.

Group	Bacteria found in marine environments	Source
Alphaproteobacteria	*Loktanella* sp. *Sulfitobacter* sp.	Burke *et al*., 2011a; Fukui *et al.*, 2014
Gammaproteobacteria	*Vibrio* sp. *Alteromona* sp. *Pseudoalteromona* sp. *Marinomona* sp. *Shewanella* sp. *Glaciecola* sp. *Oceanospirillum* sp. *Colwellia* sp.	Giovannoni and Rappé, 2000; Holmstrom *et al*., 2002; Wang *et al*., 2008; Singh *et al*., 2011; Nasrolahi *et al*., 2012
Betaproteobacteria	*Methylophilus* sp.	Megan *et al*., 2012
Bacteroidetes	*Zobellia* sp. *Krokinobacter* sp. *Nonlabens* sp. *Lewinella* sp *Ulvibacter* sp	Patel *et al*., 2003; Choi *et al*., 2007; Khan *et al*., 2007; Oh *et al*., 2009; Burke *et al*., 2011a; Baek *et al*., 2014
Cyanobacteria	*Blennothrix* sp. *Lyngbya* sp.	Clark *et al*., 2008
Firmicutes	*Bacillus* sp. *Salinicoccus* sp. *Staphylococcus* sp.	Wiese *et al*., 2009; da Silva *et al*., 2013
Actinobacteria	*Brevibacterium* sp. *Dermacoccus* sp. *Micromonospora* sp. *Micrococcus* sp. *Williamsia* sp. *Streptomyces* sp.	Pathom-aree *et al*., 2006; Bull *et al*., 2007; da Silva *et al*., 2013
Planctomycetes	*Blastopirellula* sp. *Rhodopirellula* sp. *Planctomyces* sp.	Schlesner *et al*., 2004; Ward *et al*., 2006; Bondoso *et al*., 2014

For the measurement of the bacterial community structure by culture-independent methods, some indices have been used. Particularly the Shannon index, the evenness index, derived from it, and Simpson's dominance index (Shannon and Weaver, 1963; Dunbar *et al*., 1999; McCaig *et al*., 1999; Cho and Kim, 2000). The Shannon index (*H'*) has the advantage to consider both the richness, *i.e.* the number of different species in the sample, and also, the dominance of some species. This index is suggested as one of the most appropriated methods to study diversity among different communities (Hill *et al*., 2003).

1.2.1. The phylum Planctomycetes

The phylum *Planctomycetes* is a group of bacteria that exhibit unusual characteristics some of which are shared with eukaryotic cells (Lage, 2013). Normally, they appear in low abundance in samples (Rusch *et al*., 2007). They were found in a variety of ecosystems demonstrating a cosmopolitan distribution (Chouari *et al*., 2003; Buckley *et al*., 2006; Bondoso *et al*., 2011; Fuchsman *et al*., 2012; Sheng *et al*., 2012) and have the capacity to adapt to the extreme environments such as acidic habits, extreme saline habitats, thermophilic habitats, *etc* (Giovannoni *et al*., 1987; Drees *et al*., 2006; Byrne *et al*., 2009; Li *et al*., 2010; Bernhard *et al*., 2012, Ivanova and Dedysh, 2012; Urbieta *et al*., 2012; Tang *et al*., 2013). Furthermore, planctomycetes also appear associated with eukaryotic hosts, such as macroalgae where they are common inhabitants in the biofilm of the epibacterial community (Longford *et al*., 2007; Bengtsson *et al*., 2010; Burke *et al*., 2011a; Lachnit *et al*., 2011; Lage and Bondoso, 2011; de Oliveira *et al*., 2012; Hollants *et al*., 2013; Bondoso *et al*., 2014b; Miranda *et al*., 2013). These recently confirmed associations allowed the discovery of novel planctomycetes taxa (Fukunaga *et al*., 2009; Bondoso *et al*., 2014a, 2015).

1.3. Microbial biofilms

In aquatic environments microorganisms are capable of living using different strategies. In addition to the capacity to live as individual cells in aqueous suspensions, microorganisms can colonize surfaces by the formation of the so-called biofilms. The attachment in solid surfaces and consequently the formation of biofilms is favoured by humid or aqueous environments (Weinberger, 2007). A biofilm can be defined as an assemblage of microbial cells that is irreversibly associated with a surface of living or non-living materials enclosed in an extracellular polymeric substance (EPS) matrix (Donlan, 2002) mainly composed of high-molecular weight polysaccharides. EPS matrix may account for 50% to 90% of the total organic carbon and controls the

physical properties of biofilms. The community of microbial cells can be structured and enhanced or reduced by this substance (Decho, 2000). The association of one organism growing on the surface of another is referred to as epibiosis (Bengtsson, 2011) and advantages and disadvantages may occur for both organisms. Algae, corals and sponges are some examples of eukaryotes that harbour complex epiphytic microbial communities and are susceptible to biofilm formation (Olsan and Kellogg, 2010). A number of observed host-associated microbial communities tend to be very host-specific, generally stable and normally distinct from planktonic communities in the surrounding water column (Erwin *et al*., 2011). These communities are composed by a variety of organisms such as bacteria, fungi, unicellular algae such as diatoms, and protozoa (Marshall and Bowden, 2000; Maki, 2002; Goecke *et al*., 2010). Bacteria are one of the first colonizers of the surface usually due to its high abundance in seawater (Dang and Lovell, 2000). Biofilms represent a common adaptation, perhaps even a life stage or style, of bacteria (Costerton *et al*., 1995) and the bacterial number observed in biofilms is significantly higher than in the planktonic life form (Robinson *et al*., 2010). An important feature of these epiphytic associations is that, the microbial assemblages are involved in important metabolic transformations modulating this association. In some cases, the microbial biofilm can influence the host promoting its metabolic functions such as nutrition or reproduction (Goecke *et al*., 2010; Kazamla *et al*., 2012).

1.4. Bacterial communities associated with macroalgae

In aquatic environments where macroalgae live, diseases, parasitism, epibiosis and biofouling are quite common phenomena (Harder, 2009). Thus, macroalgae are important key components in marine ecosystems providing microhabitats for many organisms. The occurrence of the macroalgae in the photic zone makes them susceptible to epibiosis because the conditions are optimal for microbial growth, namely of phyto- and zoo- species, allowing them to survive without environmental stress (Nys *et al*., 1995; Potin *et al*., 2002; Burke *et al*., 2011a). Bacteria are considered the primary colonizers of algal surfaces with a high complexity of interactions, followed by diatoms and fungi (Qian *et al*., 2007, Lam *et al*., 2008; Burke *et al*., 2011a).

Macroalgae produce organic material, such as organic carbon, providing a rich habitat in nutrients for bacteria and other microorganisms (Lane and Kubanek, 2008). The symbiotic association can protect the macroalgae from UV radiation (Koch and Brandt, 2003) and promote nitrogen fixation (Thevanatan *et al*., 2000). Additionally, bacteria may supply vitamins and/or growth regulators to the algae (Croft *et al*., 2006;

Kazamla *et al*., 2012). It was proved that some molecules produced by bacteria determine zoospores settlement in *Ulva* species (Joint *et al*., 2000) or their liberation in *Acrochaetium* (Weinberger *et al*., 2007) and *Gracilaria* species (Singh, 2013). On the other hand, when these associations are harmful for macroalgae, bacteria may provoke diseases, tissue necrosis, growth and photosynthesis reduction (Armstrong *et al*., 2000; Vairappan *et al*., 2001). Bacterial community can also affect the host by the production of a variety of toxins, digestive enzymes, inhibitors and waste products (Ivanova *et al*., 2002). However, the macroalgae developed, overtime, strategies to defend their surfaces against bacteria (Wahl, 2008; Steinberg and de Nys, 2002).

The first studies about bacteria associated to macroalgae surface dates back to before 1875 (Johansen *et al*., 1999). Even though the studies about these associations are still scarce, the utilization of the classical molecular tools associated with new tools designed as next-generation sequencing (NGS) techniques, have enabled to increase our knowledge about bacterial-algal interactions (Lachnit *et al*., 2009). Recently, a variety of symbiotic, pathological and opportunistic interactions between macroalgae and bacteria are being discovered (Goecke *et al*., 2010). Novel described bacterial species and genera were isolated from some common algae such as *Ulva*, *Porphyra*, *Fucus* and *Saccharina* (Goecke *et al*., 2013).

Regarding the diversity of bacteria found in macroalgae surfaces, some studies showed that different macroalgae species in the same location maintain different bacterial communities (Lachnit *et al*., 2009; Nylund *et al*., 2010), and the same macroalgal species from different locations have high similarities in the composition of the associated microbial communities (Staufenberger *et al*., 2008; Lachnit *et al*., 2009; Sneed and Pohnert, 2011).

1.5. Chemical interactions between macroalgae and bacteria

1.5.1. Quorum sensing – signalling

The cooperative activities and physiological processes between bacteria are regulated through a mechanism called quorum sensing (QS), in which bacterial cells communicate with each other by releasing, sensing and responding to small diffusible signal molecules. Cell-to-cell communication signals have important effects in the formation and organization of bacterial population density that contribute to the structure of biofilms, stress resistance and production of secondary metabolites (Davies *et al*., 1998; Parsek and Greenberg, 2000; Sauer *et al*., 2002; Paul and Ritson-Williams, 2008; Dobretsov *et al*., 2009). These signals are produced and excreted with

feedback regulation, and the detection of these signals eventually leads to global changes in bacterial gene expression (Waters and Bassler, 2005).

The QS in the bacteria have been generally divided into at least three classes: LuxI/LuxR–type quorum sensing in Gram-negative bacteria, which uses acyl-homoserine lactones (AHL) as signal molecules; oligopeptide-two-component-type quorum sensing in Gram-positive bacteria, which uses small peptides as signal molecules; and luxS-encoded autoinducer 2 (AI-2) quorum sensing in both Gram-negative and Gram-positive bacteria (Miller and Bassler, 2001; Federle and Bassler, 2003; Waters and Bassler, 2005).

In marine bacteria, AI-2 group of signalling molecules are important in the metabolic transformation carried out by *LuxS* enzyme that regulates the genes responsible for bioluminescence, formation of biofilms, virulence, and antibiotic production (Chen *et al*., 2002; Miller *et al*., 2002; Henke and Bassler, 2004; Miller *et al*., 2004; Bodor *et al*., 2008). This enzyme was found in *Vibrio harveyi* and since then, the *LuxS* has been identified in over 50 different bacterial species (Schauder *et al*., 2001; Winzer *et al*., 2002; Xavier and Bassler, 2003; Vendeville *et al*., 2005). As referred by Atkinson and Williams (2009), the “cross-kingdom sensing of QS signal molecules may therefore constitute an adaptive survival strategy, enabling bacteria and eukaryotes to monitor their surroundings and adjust their behaviour in response to environmental challenge and population flux”.

1.5.2. Biotechnological potential – secondary metabolites

During the last decades, cultivable microorganisms have been used as a “*pool*” of natural product drug discovery and have provided unique compounds with chemical structures that have direct application in curing diseases. The discovery of novel metabolites has been focused on marine microorganisms because the enzymes produced by these organisms are more potent biochemically and stable than those derived from plants and animals (Bull and Ward, 2000; Kin, 2006). As referred by Fusetani in 2000, the search of new drugs from marine organisms resulted in the isolation of approximately 10000 metabolites. Nowadays these organisms are viewed as sources for therapeutic agents used for treatment of cardiac, respiratory and gastrointestinal diseases (Laport *et al*., 2009). Furthermore, some evidences indicate that many bioactive compounds previously reported in marine animals and plants were in fact produced or metabolised by the associated microorganisms (Unson and Faulkner, 1993; Schupp *et al*., 1999; Davidson *et al*., 2001; Luesch *et al*., 2001; Proksch *et al*., 2002; Penesyan *et al*., 2010). In their competition for survival, bacteria

associated with other organisms produce antimicrobial and antifouling substances (Laport *et al*., 2009) demonstrating to be a rich source of bioactive compounds (Bull and Stach, 2007; Egan *et al*., 2008; Graça *et al*., 2013, 2015). *Streptomyces*, *Alteromonas*, *Pseudoalteromonas*, *Roseobacter* and *Actinomyces* are examples of the many genera of bacteria that produced bioactive compounds (Okazaki *et al*., 1975; Fenical, 1993; Wagner-Dobler *et al*., 2002).

Secondary metabolites are the substances that mediate many of these host-microbe associations in the ocean environment (Lane *et al*., 2010). Moreover, the competition for space, nutrients and defence strategies are responsible for the production of many natural products by microorganisms (Armstrong *et al*., 2001). Polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) are usually involved in the production of bioactive secondary metabolites (Donadio *et al*., 2007; Foerstner *et al*., 2008). PKSs type I are large, multifunctional enzyme complexes where intermediates translate along modules. Other PKSs have been described showing that the diversity of these systems is greater than previously recognized (Shen, 2003). The NRPS are involved with the production of small peptides. PKSs and NRPS share a similar model of biosynthesis. Both are created on modular enzymatic assembly lines, with their structural diversity governed by optional enzymes within the enzyme complexes (Walsh, 2004).

1.6. Genomic studies

Many studies of microbial communities are done, at the genetic level, without the isolation and culture of microorganisms, avoiding thus the limitation imposed by this method (Amman *et al*., 1995). For this purpose, the new molecular techniques developed have the aim to analyze the microbial structure and diversity of environmental samples and monitor changes in microbial communities (Muyzer and Smalla, 1998; Lyautey *et al*., 2005).

1.6.1. Denaturing gradient gel electrophoresis (DGGE)

The first study applied to the field of microbial ecology was developed by Muyzer and colleagues (1993). DGGE technique is usually used for detecting the phylogenetic “fingerprint” of diverse organisms by the separation of the double-stranded DNA PCR products of similar length but with different sequence composition. This separation is possible due to the denaturing gradient (urea and formamide) used in the polyacrylamide gel that cause partial denaturation of the DNA templates. In the 5’-end of one of the primers it is attached a GC-clamp (a GC-rich sequence usually 30-

50 nucleotides) to stop the complete dissociation of the double-stranded DNA (Muyzer and Smalla, 1998; Muhling *et al*., 2008; Pollet *et al*., 2011). After DNA separation in the gel, the analysis consists in the evaluation of the number, precise position and intensity of the bands which give an estimation of the number and relative abundance of numerically dominant ribotypes in the sample. Profiles are analyzed on the basis of their banding pattern (presence/absence and intensity of bands) allowing a rapid comparison of the different bacterial communities (Zoetendal *et al*., 2001). The individual bands in the gel can also be excised, reamplified and sequenced to obtain phylogenetic information (Thakur *et al*., 2008). In the study of marine bacteria, DGGE fingerprinting has been applied for many purposes, such as picoeukaryotic assemblages (Schauer *et al*., 2000; Díez *et al*., 2001), epiphytic bacteria of coral species (Morrow *et al*., 2012), sponges (Li *et al*., 2007; Thiel *et al*., 2007) and macroalgae (Ohkubo *et al*., 2006; Bondoso *et al*., 2014). Furthermore, DGGE approach allows to study seasonal and spatial variations of bacterial communities (Murray *et al*., 1998; Riemann *et al*., 1999; Riemann and Middelboe, 2002; Kan *et al*., 2006) and has revealed that bacterial communities in association with algae diverge from planctonic communities (Burke *et al*., 2011a; Goecke *et al*., 2013).

1.6.2. Next generation sequencing - Pyrosequencing

Pyrosequencing is one of the next generation sequencing techniques that permits to increase in a great detail the knowledge of microbial communities, without isolation and culture methods. This approach, based on sequencing by-synthesis principle, consists on the luminometric detection of inorganic pyrophosphate (PPi) through the action of four enzymes (DNA Polymerase I, ATP sulfurylase, Luciferase and Apyrase) in a cascade of reactions that incorporate nucleotides and produce a detectable light signal (Fig. 1). When a nucleotide is introduced in the DNA-strand, pyrophosphate is released, ATP is generated which is used for the conversion of the luciferin to oxyluciferin with generation of visible light in amounts proportional to the produced ATP (Gharizadeh *et al*., 2007; Ahmadian *et al*., 2006). The emerging of this technique allows the reducing of some limitations associated with Sanger sequencing, commonly used since 1977 (Sanger *et al*., 1977; Gharizadeh *et al*., 2007).

Pyrosequencing includes advantages, comparatively to the other sequencing methods, in terms of accuracy, flexibility, parallel processing, automatization and it does depend of labeled primers, labeled nucleotides, and gel electrophoresis (Ronaghi, 2001). Moreover, it allows to sequence short DNA sequences (at least 20 bases), and provides numerous applications such as sequencing of whole genomes, determination

of the known as well as unknown polymorphic positions, comparisons of multiple strains of bacteria and quick detection of point mutations responsible for antibiotic resistance (Ahmadian *et al*., 2006; Moder *et al*., 2007).

In this way, the application of pyrosequencing technology has revolutionized the understanding about communities' structure of bacteria in marine environment and increases the discovery of novel species which are not possible to be isolated by cultivation dependent methods.

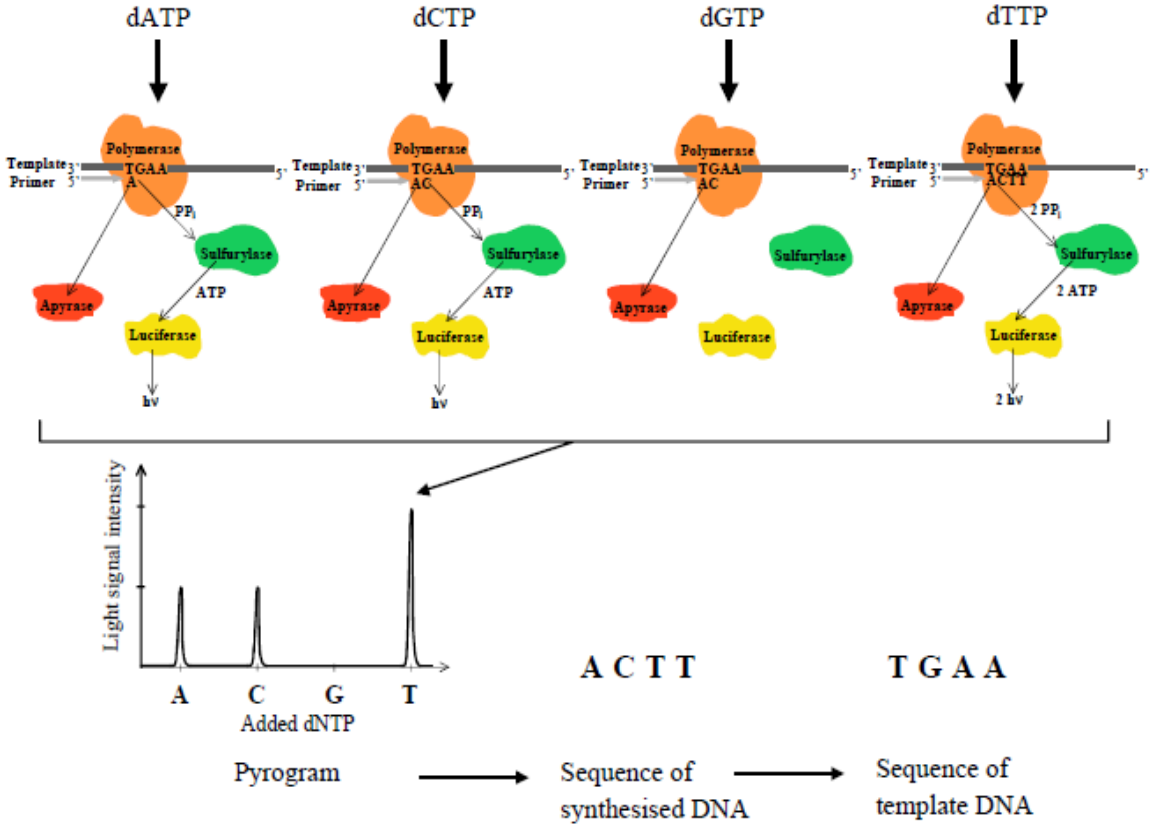


Figure 1 - Schematic representation of the pyrosequencing enzyme system. If the added dNTP forms a base pair with the template, Polymerase incorporates it into the growing DNA strand and pyrophospate (PPi) is released. ATP Sulfurylase converts the PPi into ATP which serves as substrate for the light producing enzyme Luciferase. The produced light is detected as evidence of that nucleotide incorporation has taken place (Adapted from Ahmadian *et al*., 2006).

2. Objectives

The main aim of the present work is the study of the epiphytic bacterial diversity associated with *Ulva* sp., *Porphyra dioica*, *Sargassum muticum* through culture-dependent and independent methods. Culture-dependent methods include isolation, cultivation in pure culture and identification through the analysis of the 16S rRNA gene. Seasonal variations of bacterial communities of macroalgae and surrounding water were analyzed by two independent methods: Denaturing Gradient Gel Electrophoresis (DGGE) and Next Generation Sequencing (NGS) technology. Another aim was to explore the biotechnological potential of the isolated bacteria through the search of polyketide synthase and nonrinosomal synthetase genes, which are important in the production of secondary metabolites. Potential ecological interaction between macroalgae and the different isolated bacteria was assessed through the analysis of *luxS* gene responsible for quorum sensing signalling.

3. Material and Methods

3.1. Biological material

The specimens of macroalgae were harvested at low tide from the intertidal rock platform at Luz beach, in the coast of Porto, Atlantic Ocean, Portugal (41º18'North, 8º44'West). To study the variation of microorganism's community associated to the surface of macroalgae, samples of the four seasons were collected during one year period. Specimens of each macroalgae, *Ulva* sp., *Porphyra dioica* and *Sargassum muticum* (Fig. 2) were removed and transferred into separate sterile plastic bags. One litter of the surrounding seawater was also sampled in a sterile bottle. Samples were immediately transported to the LEMUP (Laboratory of Microbial Ecophysiology of University of Porto) laboratory. Description and designation of the samples are referred in Table 2.

Microscopic observation of the macroalgae biofilm was assessed in an AxioCam MRc optical microscope and in a Phenom ProX scanning electron microscope.

Table 2 – Designation of each abbreviation used in samples for all seasons.

Samples	Description (Species; Season; Study area)
UAP	*Ulva* sp.; Autumn; Porto
PAP	*Porphyra dioica*; Autumn; Porto
SAP	*Sargassum muticum*.; Autumn; Porto
H2OAP	Sea water; Autumn; Porto
UWP	*Ulva* sp.; Winter; Porto
PWP	*Porphyra dioica*; Winter; Porto
SWP	*Sargassum muticum*.; Winter; Porto
H_2OWP	Sea water; Winter; Porto
USpP	*Ulva* sp.; Spring; Porto
PSpP	*Porphyra dioica*; Spring; Porto
SSpP	*Sargassum muticum*.; Spring; Porto
H_2OSpP	Sea water; Spring; Porto
USP	*Ulva* sp.; Summer; Porto
PSP	*Porphyra dioica*; Summer; Porto
SSP	*Sargassum muticum*.; Summer; Porto
H_2OSP	Sea water; Summer; Porto

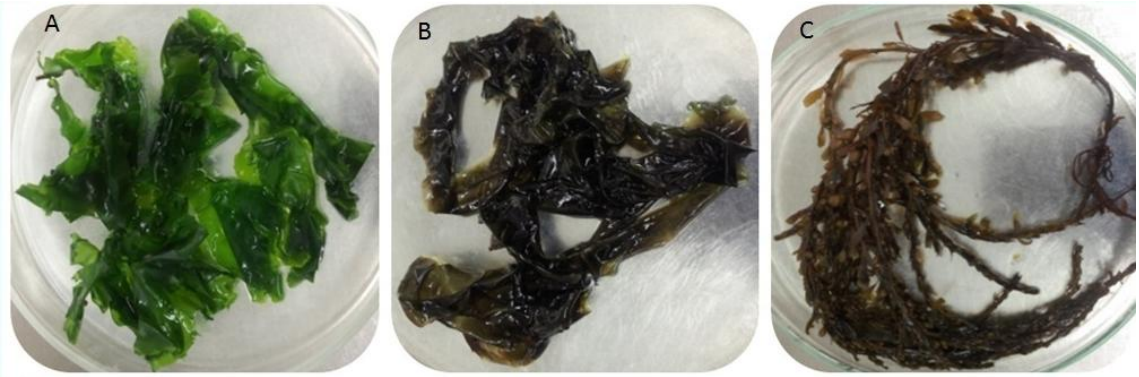


Figure 2 – Different species of macroalgae used in this study. **A** – *Ulva* sp., **B** – *Porphyra dioica* and **C** – *Sargassum muticum.*

3.2. Isolation of microorganisms

Isolation of the microorganisms was done from the samples collected in autumn. In the laboratory, the samples were manipulated under aseptic conditions, inside a flow chamber. Portions from each macroalgae were washed three times with sterile sea water, to remove non-associated bacterial cells and other small particles. One gram wet weight of each sample was macerated in 10 ml sterile natural sea water with sterile glass-beads and serially diluted (10^0, 10^{-2}, 10^{-4} and 10^{-6}). Thereafter, from each dilution, 100 µl were spread in eight selected isolation marine agar media, referred below. The cultures were incubated in the dark at 25ºC and presence of growth was checked daily. Distinct colony morphotypes were identified (colour, size, texture and shape) in a dissecting microscopy and transferred to the respective media for isolation. The pure bacterial cultures were cryopreserved in seawater supplemented with 20% glycerol at -80ºC.

The marine media used for bacteria isolation in this study were Marine Agar (MA) (Becton Dickinson), Medium F (MF) (Li *et al*., 2007), modified M13 Medium (Lage and Bondoso, 2011), *Actinomycetes* Medium (Zhang and Zhang, 2011), *Bacillus* Medium (HiCrome™), BG11 agar (HIMEDIA®) for the cultivation and maintenance of cyanobacteria, Starch Marine (HIMEDIA®), modified Myxobacterial Medium (Zhang *et al*., 2013) without cycloheximide, and for isolation of cellulose-degrading myxobacteria we used slices of filter paper which were placed on top of the same medium. For quantification of the Colony Forming Units (CFU), three different plates with MA medium for each dilution of each macroalgae were used.

Moreover, for the isolation of *Planctomycetes*, portions of macroalgae were placed in different plates containing modified M13 medium that was supplemented with streptomycin, ampicillin and pevaryl, and incubated in the dark at 25ºC according to Lage and Bondoso (2011). This procedure was repeated in all seasons.

Seawater samples were filtered through a 0.22 µm pore filter for DGGE and pyrosequencing analyses (described below), and temperature, salinity, conductivity and pH were measured.

3.3. Molecular Analysis

3.3.1. Community DNA extraction and amplification

The genomic DNA of bacteria isolated from the specimens were extracted using the E.Z.N.A. bacterial KIT from OMEGA, according to the manufacturer's instructions. The taxonomic identification of bacteria isolated was based on the analysis of the 16S rRNA gene. This gene was amplified from the extracted DNA with the universal primers, 27F and 1492r (Lane, 1991) in 50 µl of PCR mixture (1 x Green GoTaq® Flexi Buffer; 1.5 mM $MgCl_2$; 1 unit of GoTaq® Flexi DNA Polymerase; 200 µM of each deoxynucleoside triphosphate (dNTPs); 2 µM of each primer). Two µl of DNA template were used for the PCR reaction. The PCR program was performed in a MyCycler™Thermo Cycler (Bio-Rad) and amplification conditions comprised initial denaturing step of 5 minutes at 95 ºC; 30 cycles of 1 minute at 94 ºC; 1 minute at 52 ºC, 90 seconds at 72 ºC and a final extension of 5 minutes at 72 ºC. PCR products were visualized after electrophoresis in a 1.2% agarose gel stained with Roti Safe (Roth) in 1 x Tris Acetate and EDTA (TAE) buffer (OMEGA).

3.3.2. Identification and phylogeny analysis

Amplification products were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced for the 16S rRNA gene at Macrogen. The sequences were edited and checked manually using CHROMAS 2 (Goodstadt and Ponting, 2001) correcting possible errors in chromatograms. The corrected sequences were assembled and consensus of the strains was constructed in ProSeq v2.91 and Vector NTI 11.5.3.

Alignment of all consensus sequences was performed using MEGA 6 (Molecular Evolutionary Genetics Analysis) software, that permits to infer overtime the molecular evolutionary between genes, genomes and species (Tamura *et al.,* 2013). The construction of the phylogenetic tree was performed using calculation methods (maximum likelihood – ML) in MEGA 6, applying General Time Reversible model and Gamma distributed with Invariant sites (G+I). The aligned sequences were compared in GenBank using a Basic Local Alignment Search Tool (BLAST). Different phylotypes

were considered based on a 97% 16S rRNA gene threshold (Stackebrandt and Goebel, 1994).

3.3.3. Biotechnologic potential - search of polyketide synthase and nonribosomal peptide syntethase genes

The presence of the genes PKS-I and NRPS involved in the production of secondary metabolites was screened in all bacteria isolated from the specimens of macroalgae. Amplification of the extracted DNA was achived with MDPQQRf and HGTGTr (Kim *et al*., 2005) and DKf and MTr (Neilan *et al*., 1999) primers, specific for PKS-I and NRPS genes respectively, in 25 µl of PCR mixture (1 x Green GoTaq® Flexi Buffer; 1.7 mM $MgCl_2$; 0.8 unit of GoTaq® DNA Polymerase; 0.2 mM of each dNTPs; 0.1mM of each primer and 2 µl DNA template. The same PCR program was used for the amplification of two genes in a MyCycler™Thermo Cycler (Bio-Rad). The amplification conditions consisted of an initial denaturing step of 5 min at 95 ºC; 11 cycles of 1 min at 95 ºC; 30 s at 60 ºC and 1 min at 72 ºC, with the annealing temperature reduced by 2 ºC per cycle, followed by 30 cycles of 95 ºC for 1 min, 40 ºC for 30 s and 72 ºC for 1 min with a final extension of 10 min at 72 ºC. The PCR products were visualized by electrophoresis for the presence of approximate 700 bp and 1000 bp size amplicons, for PKS-I and NRPS respectively, in a 1.2% agarose gel in 1 x TAE buffer.

3.3.4. Quorum sensing analysis - LuxS gene

The search of the luxS gene was based on Bodor *et al*., (2008), who used marine, Gram positive and negative bacteria, to analyze the potential for luxS signaling. PCR based on the amplification of luxS gene was performed using LuxS_degfor3 and LuxS_degrev4 primers (Table 3) with a highly degenerate 5' end and a specific 3' end. PCR mixture (50 µl) contained: 1 x Green GoTaq® Flexi Buffer; 3 mM $MgCl_2$; 1.25 unit of GoTaq® DNA polymerase; 1 µM of each primer; 200 µM dNTPs and 2 µl DNA template. The PCR program was conducted in a MyCycler™Thermo Cycler (Bio-Rad) and the conditions consisted in a predenaturing at 94 ºC for 1 min; 30 cycles of denaturing at 94 ºC for 10 s; annealing at 48 ºC for 30 s; elongation step at 68 ºC for 40 s; and a final extension at 68 ºC for 1 min 30s. PCR products were visualized after electrophoresis in 1.2 % agarose gel in 1 x TAE buffer.

Table 3 – LuxS primers used (forward and reverse).

Name	Sequence (5'-3')	Length	Degeneracy	Ann. Temp.	Start position
LuxS_degfor3	CATTATTAGATAGCTTTACADTNGAYCAYA	30 bp	48	48 ºC	4
LuxS_degrev4	AGCGAGTGCATCTGATAAGWNCCRCAYTS	29 bp	64	48 ºC	410

3.3.5. DGGE fingerprinting of seasonal variation in the epiphytic community

For the analysis of DGGE bacterial profiles an extraction kit was used, the PowerSoil® DNA Isolation Kit, that allows the isolation of microbial genomic DNA from soil and other environmental samples. In this case, the samples used were the homogenized from each macroalgae and the filtrate from sea water sampled.

To compare the profiles of bacterial communities of all seasons (macroalgae and seawater), PCR based on the analysis of 16S rRNA gene were performed using encoding primers GC-358F (5′-CCT ACG GGA GGC AGC AG-3′ with a GC-clamp at the 5´ end) and 907r (5′-CCG TCA ATT CMT TTG AGT TT-3′) in 50 µl of PCR mixture: 1x Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 µM of each dNTPS, 0.5 µM of each primer, 0.75 units of GoTaq® Flexi DNA Polymerase and 5 µl of DNA as template. These primers are specific for bacteria and the DNA fragments produced have around 550 bp.

PCR amplification was performed in a MyCycler™Thermo Cycler (Bio-Rad), that consisted in a initial denaturing step of 94 ºC for 5 minutes; 10 cycles of 1 minute at 94 ºC; 1 minute at decreasing temperature with each cycle starting at 65 ºC and ending at 55 ºC, 3 minutes at 72 ºC; 20 cycles of 1 minute at 94 ºC; 1 minute at 55 ºC; 3 minutes at 72 ºC and a final extension of 10 minutes at 72 ºC. PCR products were separated by electrophoresis on 1.2% agarose gel in 1 x TAE buffer.

The concentrations of the samples were calculated with the Thermo Scientific™ µDrop™ Plate that use a photometric measurement to quantify the nucleic acids in a sample. For quantification 2 µl of sample and standard (Green GoTaq® Flexi Buffer) were used.

After this procedure, about 600 ng of PCR products from each mixture were loaded in a DGGE gel and run at 60 °C at constant voltage of 120 volts for 16 hours in a DGGEK-2401-220 system (CBS Scientific Company). The 6% acrylamide gel with a linear gradient of denaturing conditions (100% denaturant agent is 7 M urea and 40% deionized formamide) ranged from 40 to 60%, 40 to 70% and 40 to 80% (based on Díez *et al*., 2001).

The ladder was prepared with a mixture of PCR products of bacterial communities associated with macroalgae. Gels were stained with SYBR® Gold Nucleic Acid Gel Stain during 1 hour in 1 × TAE buffer and visualized by UV light in a ChemiDoc system (Bio-Rad).

The digitalized DGGE gel was analyzed with the QuantityOne software (Bio-Rad). This software performs an optical intensity profile through each lane, detects the bands and identifies the bands occupying the same position in the different lanes of the gel.

Matrix reports were statistical analysed using PAST 2.17 (Hammer *et al*., 2001) and cluster analysis, non-metric multidimensional scaling (nMDS), similarity percentage analysis (SIMPER), analysis of similarity (ANOSIM), all based in Bray-Curtis coefficient (Bray and Curtis, 1957) were performed. Furthermore, diversity index (Shannon-Weiner and CHAO1) was also analysed.

3.3.6. Pyrosequencing analysis

Pyrosequencing sequencing and analysis were conducted at Plymouth University. Autumn and winter samples used in this analysis were the same that were extracted for DGGE analysis. PCR amplification of the 16S rRNA V1-V2 region was conducted using the primers 338R (5'-GCW GCC WCC CGT AGG WGT-3') and 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') according to Newton and Roeselers (2012). Each PCR reaction contained 1 µl of each primer, 25 µl of MyTaq^{TM}, 23 µl of molecular grade water and 1 µl of DNA template. Thermal cycling was ferformed using a TC-512 thermal cycler in the following conditions: initial denaturating step at 94 °C for 7 min, 10 cycles at 94 °C for 30 s, touchdown of 1 °C per cycle from 62 to 53 °C for 30 s and 72 °C for 30 s. A further 25 cycles were performed at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s before a final extension for 7 min at 72 °C.

PCR products were cleaned using PCR purification columns (QIAquick PCR Purification Kit; Qiagen) prior to high-throughput sequencing. To ensure that there was sufficient DNA present, some PCR reactions were made in duplicate (UWP, PWP, SWP) and pooled into a single sample prior to cleaning.

The amplified and purified DNA from the samples was quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Prior to sequencing, the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (LifeTechnologies^{TM}) and concentrations were then adjusted to 26 pM.

Amplicons were attached to Ion Sphere Particles using Ion PGM^{TM} Template OT2 400 kit (LifeTechnologies^{TM}) according to the manufacturer's instructions.

Multiplexed sequencing was conducted using Ion Xpress^{TM}Barcode Adapters (LifeTechnologies^{TM}) and a 318^{TM} chip (LifeTechnologies^{TM}) on an Ion Torrent Personal Genome Machine (LifeTechnologies^{TM}) at the Systems Biology Centre in Plymouth University. Sequences were binned by sample and filtered within the Ion Personal Genome Machine (PGM) software to remove low quality reads.

Data were then exported as FastQ files. Taxonomic analyses of sequence reads were performance after the removal of low quality scores (Q score < 20) with FASTX-Toolkit (Hannon Lab). Sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were denoised and analyzed with QIIME (Caporaso *et al*., 2010b). Briefly, OTU (Operational Taxonomic Unit) mapping was performed using the USEARH quality filter pipeline (Edgar, 2010), to remove putatively erroneous reads (chimeras), then OTU picking was achieved with a minimum pair wise identity of 97%.

The most abundant sequence in each OTU were selected to assign a taxonomic classification based on the Greengenes database (DeSantis *et al*., 2006) using the RDP classifier (Wang *et al.,* 2007), clustering the sequences at 97% similarity with a 0.80 confidence threshold. Sequences were filtered to remove outliers and filter positions with gaps (0.95) and singletons. PyNast was used to create a multiple alignment of the representative sequences for each OTU (Caporaso *et al.,* 2010a) with minimum sequence length threshold of 150 bp and 95% identification.

Alpha diversity metrics were calculated on rarefied OTU tables with QIIME to assess sampling depth coverage using Chao1, Good's coverage, observed species, Phylogenetic Diversity and Shannon's diversity index. QIIME was also used to calculate beta diversity metrics among samples using weighted and unweighted Unifrac distances (Lozupone *et al*., 2007) and BrayCurtis similarity (Bray and Curtis, 1957).

To determine if significant differences among seasonality or macroalgae species and seawater in high-throughput data non-parametric tests were performed on the OTU relative abundance data and alpha diversity metrics. Additionally, Vegan and APE packages of R were used to analyze the beta diversity of the communities from the eukaryotic species and seawater and the effect of seasonality. Statistical significance was accepted at the $p < 0.05$ level.

4. Results and Discussion

4.1. Macroalgae associated biodiversity

4.1.1. Bacterial isolation studies

The characterization of the bacterial community associated with the three macroalgae under study, *Ulva* sp., *Porphyra dioica* and *Sargassum muticum* was done in the samples collected in Autumn through cultural methods. These algae are representatives of the three lineages of macroalgae, Chlorophyta, Rhodophyta and Heterokontophyta, that are commonly found in the North Coast of Portugal. *Ulva* and *P. dioica* are native inhabitants in this area (Oliveira, 1990; Pereira *et al*., 2001) but *S. muticum* is an invasive species (Vaz-Pinto *et al*., 2014). The three macroalgae were observed by optical and electron microscopy (OM and EM) to assess the biofilm on their surfaces (Figs 3 and 4).

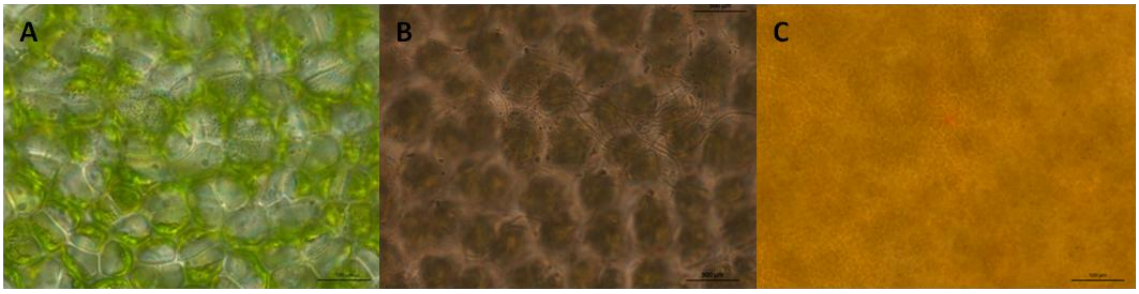


Figure 3 - Microbial biofilm obtained through optical microscopy (OM) in each macroalgae. **A** – *Ulva* sp., **B** – *Porphyra dioica* and **C** – *Sargassum muticum*.

Microbial biofilm is well visible by OM in *Ulva* sp and *P. dioica* surfaces but impossible to visualize in *S. muticum* thalus due to its width (Fig. 3). By EM the biofilm was clearly visible in the three macroalgae. *S. muticum*, however, showed zones without (Fig. 4C-1) and with (Fig. 4C-2) epibacterial community. In the kelp *Laminaria hyperborea* was also observed a highly variable density and distribution of the microbial cells on its surface (Bengtsson *et al*., 2010). *S. muticum* also revealed the presence of high abundance of diatoms (Fig. 4D-3).

A total of 245 isolates were obtained, 100 (41 %) from *Ulva* sp., 61 (25 %) from *P. dioica* and 84 (34 %) from *S. muticum*. Bacteria were obtained in all media assayed except in BG11 agar and the highest number of colonies was obtained for the three macroalgae species in the non-selective MA medium (Fig. 5). As previously referred, MA is a medium allowing the growth of a high number of isolates of heterotrophic marine bacteria (Webster *et al*., 2001, Radwan *et al*., 2010; Graça *et al*., 2015). On the contrary, more selective media, like the Actinomycetes medium, allowed low levels of

isolation. Curiously, Joint *et al.,* (2010) on an isolation study of bacterial groups from seawater of the English Channel obtained a very high number of isolates in Actinomycete medium (mainly *Gammaproteobacteria* and *Actinobacteria*) and a much lower number in MA. Seawater is a much lower organic carbon content environment when compared to biofilms. It is thus expected that a medium like MA that contains organic carbon in concentrations much higher than those found in most natural environments like seawater (Toledo *et al*., 2006), favours the growth of typically fast growing heterotrophic marine bacteria present in biofilms. The planctomycetes selective M13 medium also allowed a quite high percentage of isolation (around 8 %) which may be due to the quite high percentage of planctomycetes known to exist on macroalgae surfaces (Bengtsson and Øvreås, 2010; Lachnit *et al*., 2011).

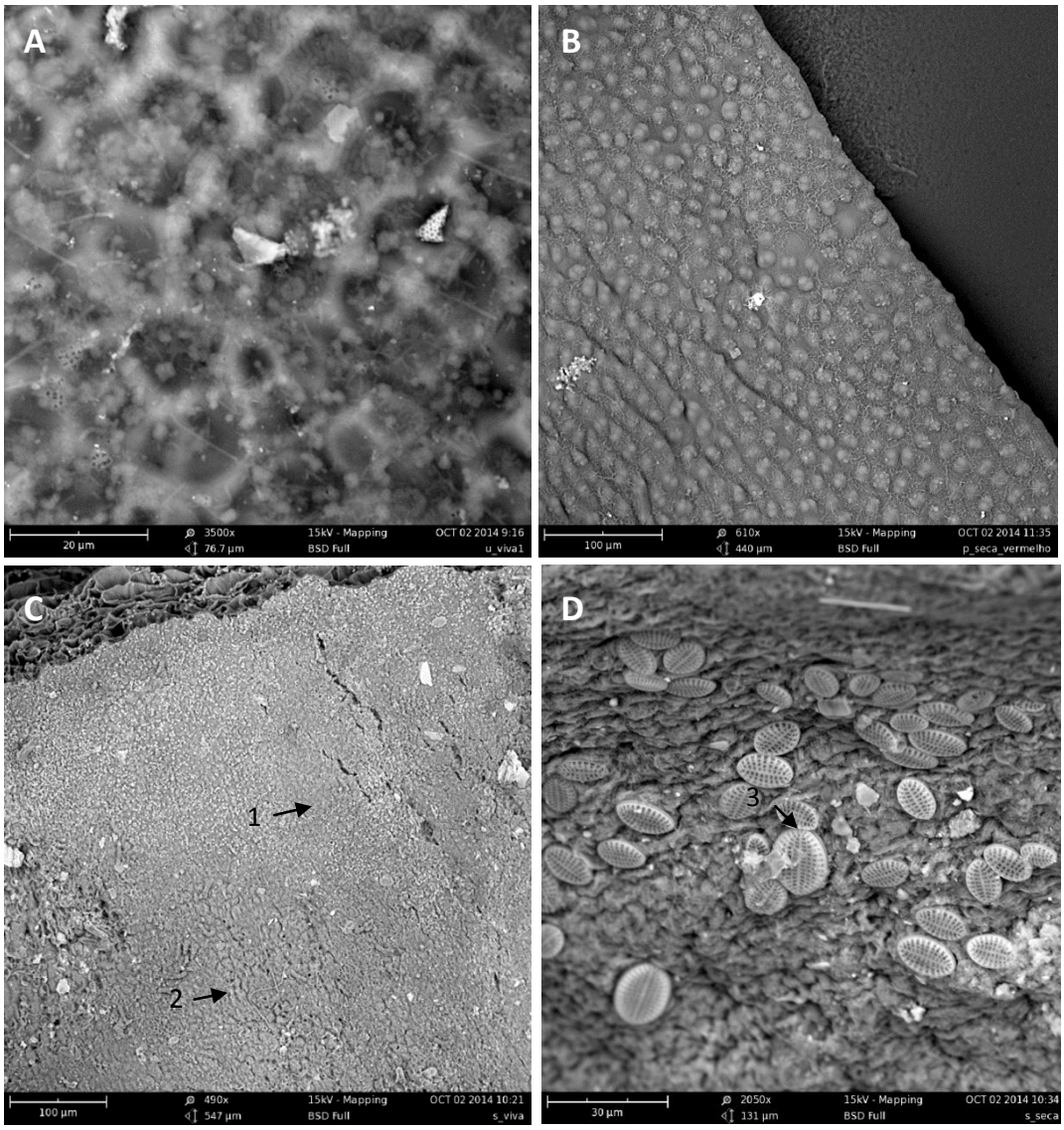
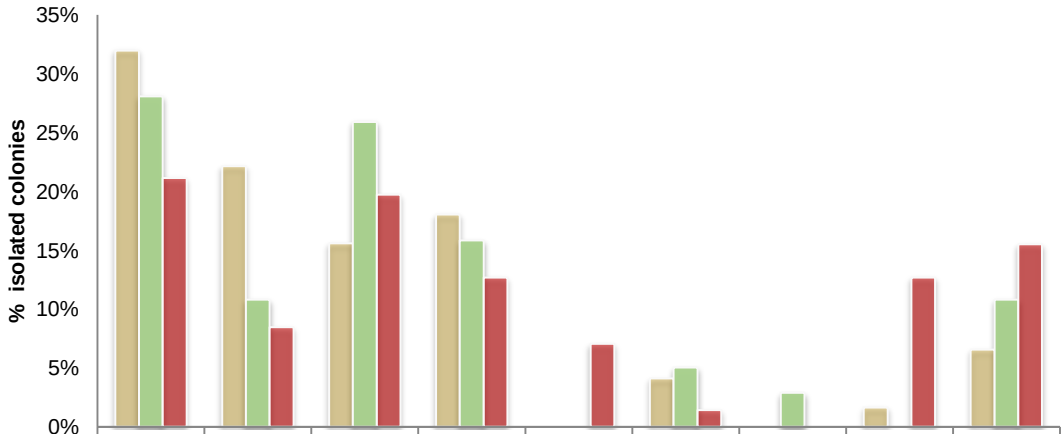


Figure 4 – Observation of the microbial biofilm, by scanning electron microscopy (SEM), associated to the macroalgae. **A** – *Ulva* sp., **B** – *Porphyra dioica*, **C** (1 – Without epibacterial community; 2 – With epibacterial community) and **D** (3 – diatoms) – *Sargassum muticum*.

In MA medium, cell concentration associated with each macroalgae was $9.03x10^3$ CFU per 1 g of wet biomass for *Ulva* sp., $2.28x10^4$ CFU per 1 g of wet biomass for *P. dioica* and 1.86 x 10^4 CFU per 1 g of wet biomass for *S. muticum*. *Ulva* sp. revealed the lowest number of associated bacteria. Beleneva and Zhukova (2006) obtained higher bacterial cell concentrations (from 1.37×10^5 to 6.14×10^5 cells per 1 g of wet biomass) on healthy brown (*Desmarestia viridis* and *Chordaria flagelliphormis*) and red algae (*Gracilaria verrucosa* and *Camphylaephora hyphaeoides*) grown on Y-K-agar (Youschimizu and Kimura, 1976). This medium has higher organic concentration then MA.



	MA	MF	ST	Myxobacterial	Myxobacterial/filter paper	Bacillus	Actinomycetes	M13	M13 portions
SAP	32%	22%	16%	18%	0%	4%	0%	2%	7%
UAP	28%	11%	26%	16%	0%	5%	3%	0%	11%
PAP	21%	8%	20%	13%	7%	1%	0%	13%	15%

Figure 5 – Number of colonies in percentage obtained from *Ulva* sp. (UAP), *P. dioica* (PAP) and *S. muticum* (SAP) sampled in Autumn in Porto for all media.

The phylogenetic identification of the bacterial isolates was, in general, only performed for the bacteria that demonstrated bioactive potential through the search of the PKS-I and/or NRPS genes due to financial constraints. Only 86 bacteria were identified by the analysis of the 16S rRNA gene sequence from the 245 microorganisms isolated (Fig. 6). Figure 7 shows the amplification of the 16S rRNA gene for different isolates.

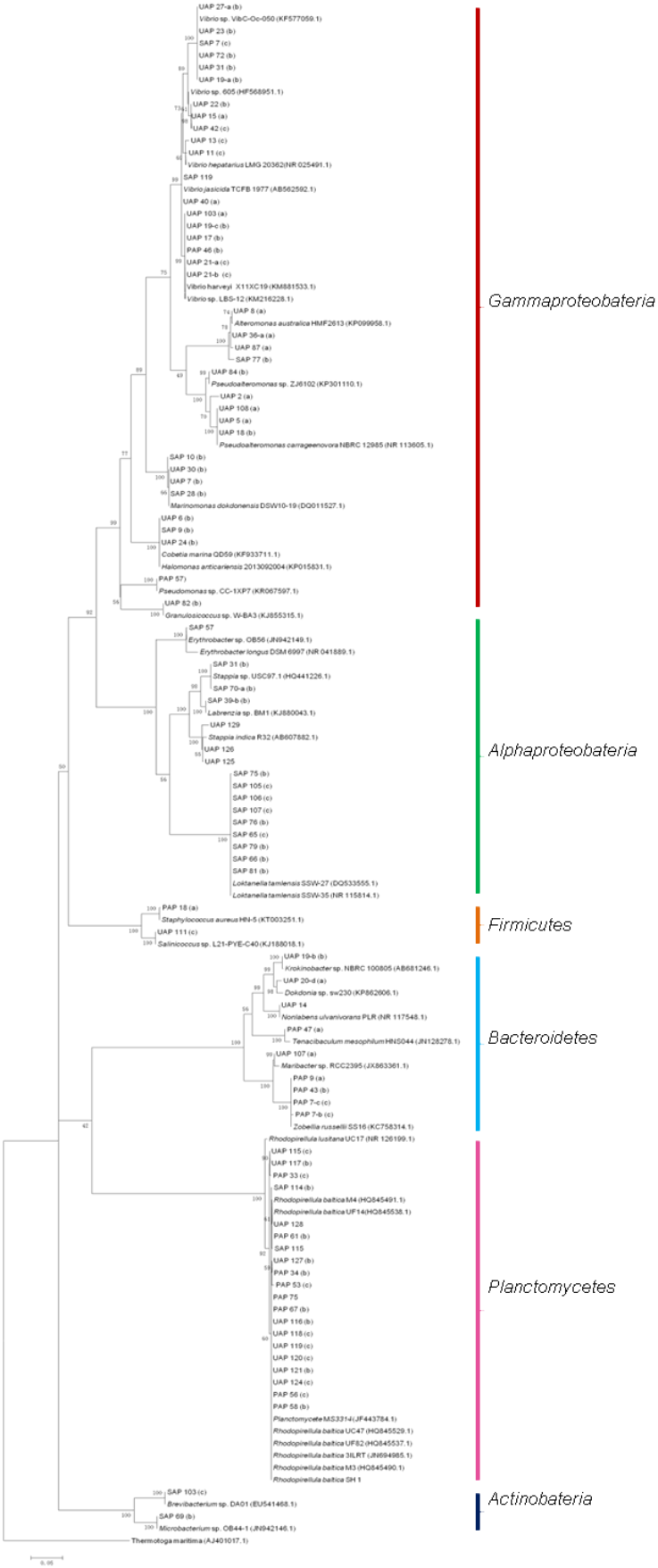


Figure 6 – Phylogenetic 16SrRNA gene tree generated by maximum-likelihood analysis based in General Time Reversible model and Gamma distributed with Invariant sites (G+I) indicating the relationship of the bacteria isolated from the three macroalgae that demonstrated bioactive potential. *Thermatoga maritima* was used as out-group. The numbers beside nodes are the percentages for bootstrap analyses; only values above 50% are shown. Bar – 0.05 substitutions per 100 nucleotides. (**a**) Presence of NRPS gene; (**b**) Presence of PKS-I gene; (**c**) Presence of both genes.

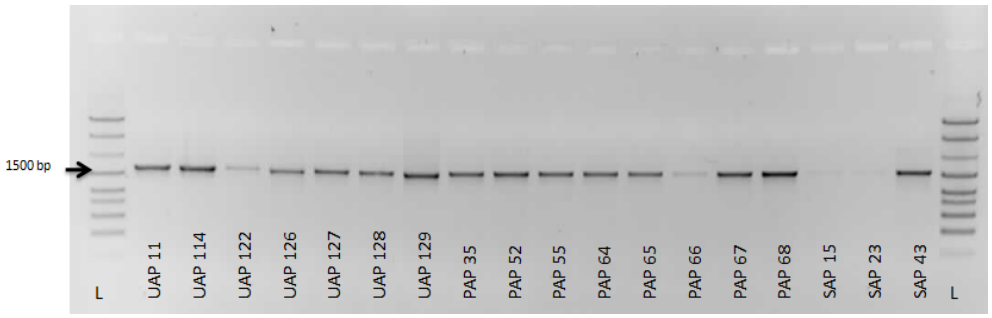


Figure 7 – Electrophoretic gel showing the 16S rRNA gene amplification in some bacteria isolated from *Ulva* sp., *P. dioica* and *S. muticum*. L – Ladder (GeneRuler^{TM} DNA Ladder Mix). For isolates designations see Table 2.

The majority of the identified bacteria belonged to the *Proteobacteria* (62 % of which 43 % from *Gammaproteobacteria* and 19 % from *Alphaproteobacteria*), followed by *Planctomycetes* (23 %), *Bacteroidetes* (*Flavobacteria* – 11 %) and *Actinobacteria* and *Firmicutes* (both 2 %) (Figs. 6 and 8).

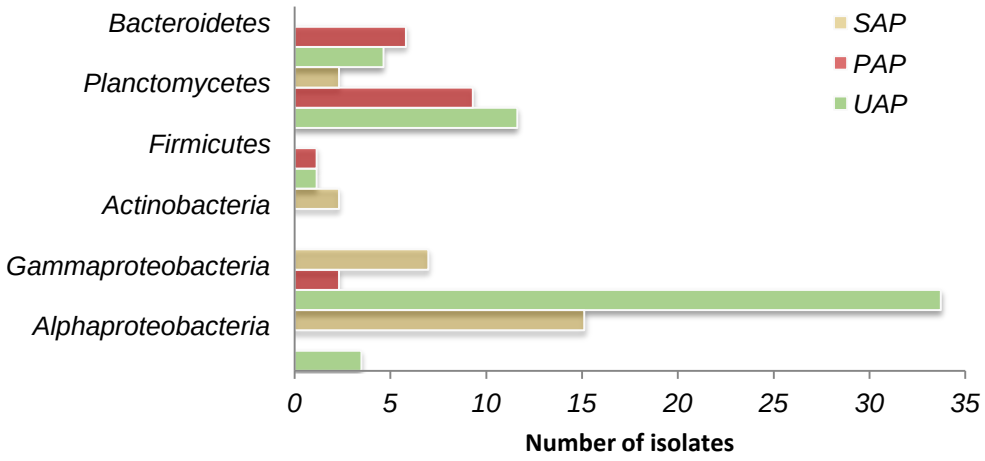


Figure 8 – Number of isolates obtained in each taxonomic group associated to the different macroalgae.

Ulva sp. harboured bacteria from *Gammaproteobacteria* (the most abundant group), *Alphaproteobacteria*, *Planctomycetes* and *Bacteroidetes*, showing the highest diversity of the three algae. In the *Gammaproteobacteria*, all the genera identified had representatives and the genus *Vibrio* was the most abundant (Figs 6 and 9). Also abundant, were the genera *Rhodopirellula* and *Pseudoalteromonas*. Relatively to *P. dioica*, *Planctomycetes* (*Rhodopirellula*) and *Bacteroidetes* (*Zobellia and Tenacibaculum*) were the two most represented phyla but also *Gammaproteobacteria* and *Firmicutes* were observed (Figs 6 and 9). In *S. muticum*, of all the groups, only *Bacteroidetes* were not present and the *Alphaproteobacteria* was the most represented one, with dominance of the genus *Loktanella* (Figs 6 and 9).

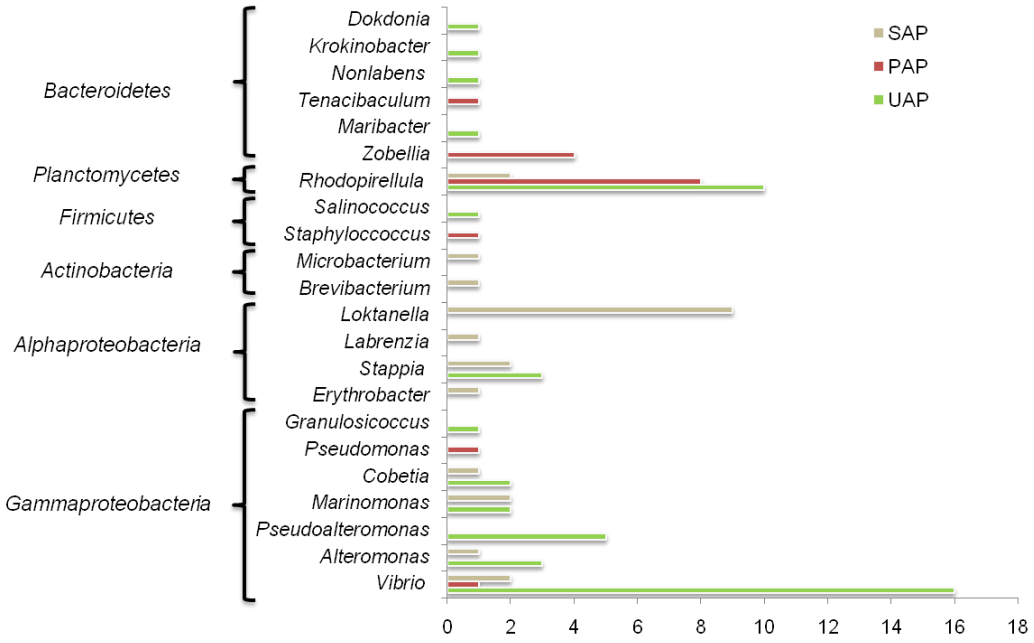


Figure 9 – Number of isolates referred by genus for each macroalgae in Autumn.

Overall, in the *Gammaproteobacteria* the genus *Vibrio* was the most abundant (51 %) but also isolates affiliated to *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Halomonas* and *Granulosicoccus* were obtained. In the *Alphaproteobacteria* the dominant genus was *Loktanella* (56 %). Also present were isolates affiliated to minor genera which were *Erythrobacter*, *Stappia* and *Labrenzia*. The *Planctomycetes* obtained were all affiliated to *Rhodopirellula baltica*. The *Bacteroidetes* were all *Flavobacteria* from the genera *Zobellia*, *Maribacter*, *Tenacibaculum*, *Nonlabens*, *Krokinobacter and Dokdonia*. In the *Firmicutes,* only the genera *Staphylococcus* and *Salinicoccus* were identified and in the *Actinobacteria* the genera *Brevibacterium* and *Microbacterium* (Figs. 6 and 9).

According to Singh and Reddy (2014) the most abundant bacteria on seaweed surfaces belong to the phyla *Proteobacteria* and *Firmicutes*. However, Wahl and collaborators (2012) and Hollants and collaborators (2013) refer that Proteobacteria and Bacteroidetes are the major lineages dominating surface communities on macroalgae. Undoubtedly in this work, *Proteobacteria* was also the most abundant identified phylum in *Ulva* sp. and *S. muticum* as well as in *Caulerpa taxifolia* (Meusnier *et al*., 2001), *Fucus vesiculosus* (Stratil *et al*., 2013), *Ulva* (Hagstrom *et al*., 2000; Patel *et al*., 2003; Tujula *et al*., 2010; Burke *et al*., 2011a, b) and three Rhodophyta (Wu *et al*., 2014). Furthermore, *Alphaproteobacteria* or *Gammaproteobacteria* are normally the major groups of marine bacteria obtained by culture methods (Wichard, 2015).

Bacteroidetes and/or *Planctomycetes* are also frequently observed in macroalgae as revealed by several studies (*Caulerpa taxifolia* - Meusnier *et al*., 2001;

Desmarestia viridis, Chordaria flagelliphormis, Gracilaria verrucosa and Camphylaephora hyphaeoide - Beleneva and Zhukova, 2006; *Enteromorpha/Ulva intestinalis* - Patel *et al*., 2003 and Lachnit *et al*., 2011; *Ulva* – Tait *et al*., 2009, *Corallina officinalis* – Huggett *et al*., 2006; *Ulva australis* - Tujula *et al*., 2010; *Porphyra umbilicalis* – Miranda *et al*., 2013; *Laminaria hyperborea* – Bengtsson *et al*., 2010). *Bacteroidetes* and Planctomycetes were also abundant in this study. Curiously, Bacteroidetes was the most abundant group in *P. umbilicales* (Miranda *et al*., 2013) and *P. yezoensis* (Wu *et al*., 2014).

Representatives of the genus *Vibrio* are natural inhabitants of aquatic environments and form symbiotic or pathogenic relationships with eukaryotic hosts (Yildiz and Vilsik, 2009). Furthermore, *Vibrio* sp. were found to be opportunistic pathogens in diseased *Porphyra* and *Laminaria* fronds (Wang *et al*., 2008). This was the most abundant genera in this work and its presence in the bacterial community of macroalgae is well established (Hollants *et al*., 2013). Other genera common inhabitants of macroalgae and also found in this study are *Alteromonas*, *Pseudoalteromonas*, *Erythrobacter*, *Loktanella*, *Staphylococcus*, *Rhodopirellula*, *Pseudomonas* and *Zobellia* (Huggett *et al*., 2006; Hengst *et al*., 2010; Hollants *et al*., 2013; Wu *et al*., 2014). *Tenacibaculum* was also isolated from several macroalgae (Suzuki *et al*., 2001; Matsuo *et al*., 2003). *Marinomonas* was observed in *Gloiopeltis furcata* (Wu *et al*., 2014). *Granulosicoccus* appeared in the red alga *Delisea pulchra*, the green alga *U. australis* (Longford *et al*., 2007), the brown alga *Fucus vesiculosus* (Lachnit *et al*., 2011) and the brown alga *Saccharina latissima* (Staufenberger *et al*., 2008). *Maribacter* spp. were isolated from the green algae *Ulva fenestrata* (Nedashkovskaya *et al*., 2004) and *Ulva australis* (Burke *et al*., 2011a) and the red algae *Polysiphonia japonica* (Nedashkovskaya *et al*., 2007). *Krokinobacter* was in *Ulvaceae* (Tujula *et al*., 2010; Burke *et al*., 2011a) and in *P. umbilicales* (Miranda *et al*., 2013). The isolate UAP 14 from *Ulva* sp. is afiliated to *Nonlabens ulvanivorans* which is described as an ulvan-degrading bacterium (Kopel *et al*., 2014). The *Actinobacteria*, *Microbacterium* and *Brevibacterium*, have been described as having defense functions for their algal hosts and *Pseudoalteromonas* spp, *Tenacibaculum amylolyticum*, *Vibrio tasmaniensis* and *Zobellia galactanovorans* were described as species that benefit the macroalgae (Hollants *et al*., 2013). *Pseudoalteromonas carrageenovora* is common in marine environments and produces carrageenases that degrade the carrageenans produced by red seaweeds (Michel *et al*., 2001). However the isolates affiliated to *P. carrageenovora* were isolated from *Ulva* sp. and not from a red alga. In Tait *et al*., (2009) study, *Ulva* were dominated by *Alphaproteobacteria*, particularly the *Rhodobacteraceae* family, and the *Bacteroidetes* family *Flavobacteriaceae*. In our

study, the *Rhodobacteraceae Loktanella* was observed in *Sargassum muticum* and the *Flavobactereaceae* were observed in *Ulva* sp. and *P. dioica*.

4.1.2. Seasonal phylogenetic analysis of *Planctomycetes*

Previous isolations performed in this laboratory have demonstrated the presence of a significant planctomycetal community associated with various macroalgae (Lage and Bondoso, 2011) which lead us to do further planctomycetes isolations that were attempted in Autumn, Winter and Spring. Isolates were obtained from the incubation of portions and macerated extracts of the macroalgae. Twenty planctomycetes were obtained in Autumn as well as in Spring. In Winter no isolation was achieved.

Phylogenetic identification was obtained through the analysis of 16S rRNA gene. Figure 10 shows 16S rDNA amplification of Spring isolates and Figure 11 is the phylogenetic tree of all the planctomycetes sequenced until now.

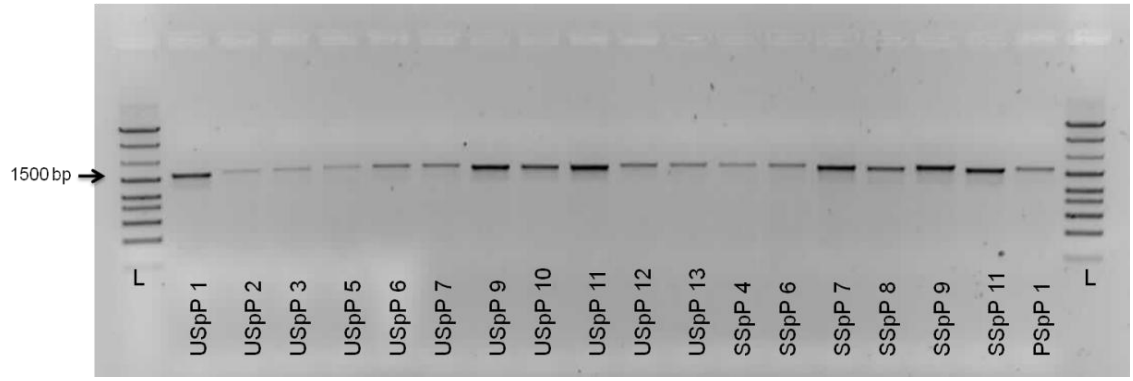


Figure 10 - Electrophoretic gel showing the 16S rRNA gene amplification of bacteria isolated in Spring from each macroalgae (*Ulva* sp., *P. dioica* and *S. muticum*). L – Ladder (GeneRulerTM DNA Ladder Mix). For isolates designations see Table 2.

All the *Planctomycetes* identified appeared in the macroalgae from the two seasons analyzed and are closely related to *Rhodopirellula baltica* (Figs. 11 and 12) in particular strains that have been previously isolated also from various macroalgae in the same sampling place (Lage and Bondoso, 2011). This result confirms the intimate association of this species with macroalgae in their epiphytic community and corroborates previously studies (Bengtsson and Øvreås, 2010; Lage and Bondoso, 2011; Bondoso *et al*., 2014).

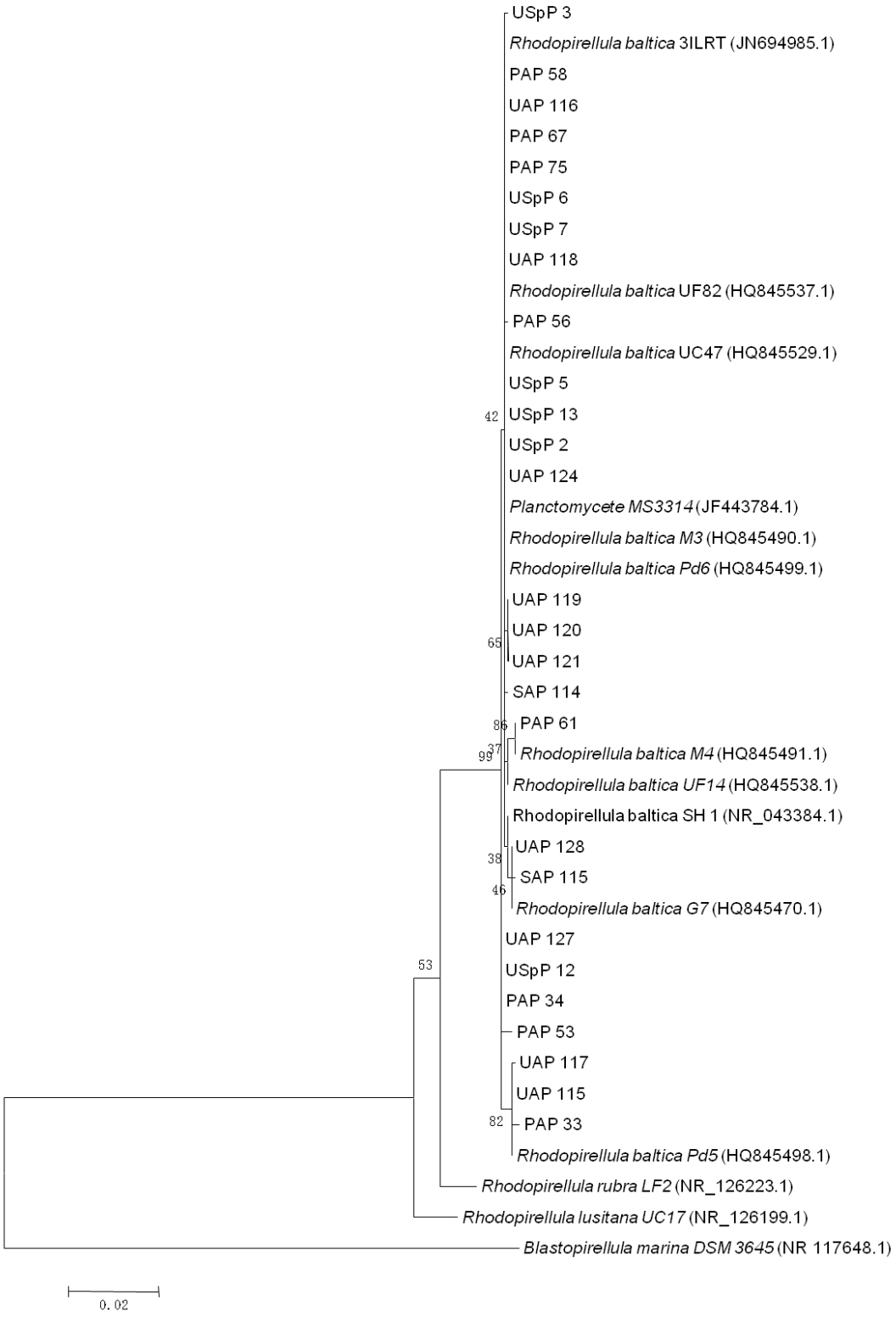


Figure 11 - Phylogenetic 16S rRNA gene tree generated by maximum-likelihood analysis based in General Time Reversible model and Gamma distributed with Invariant sites (G+I) indicating the relationship of the *Planctomycetes* isolated in Autumn and Spring seasons from the three macroalgae. *Blastopirellula marina* was used as out-group.

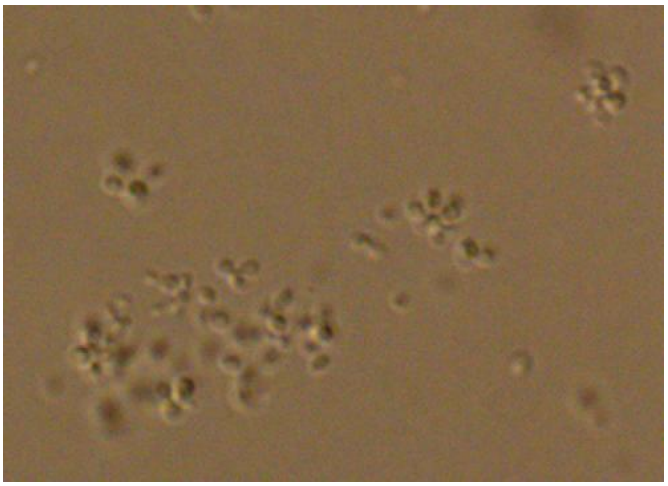


Figure 12 - OM image of a planctomycetes isolate obtained from *Ulva* sp. in Spring. The characteristic rosette formation of *Rhodopirellula* spp. is well evident.

After the alignment of the sequences and the comparison in GenBank, using BLAST, strains USpP 1, USpP 9, USpP 10 and USpP 11 were not included in the phylogenetic tree, because they belong to the *Alphaproteobacteria Erythrobacter* genus (99% identity with *Erythrobacter longus)*. These isolates were initially thought to be planctomycetes due to the reddish colour of their colonies and their growth in the planctomycetes selective medium. It must be noticed that isolate SAP 57 from Autumn is also an *Erythrobacter* closely related to the same species. *E. longus* was described by Shiba and Simidu in 1982 after several isolates have been obtained from the macroalgae *Enteromorpha linza* and *Porphyra* sp.

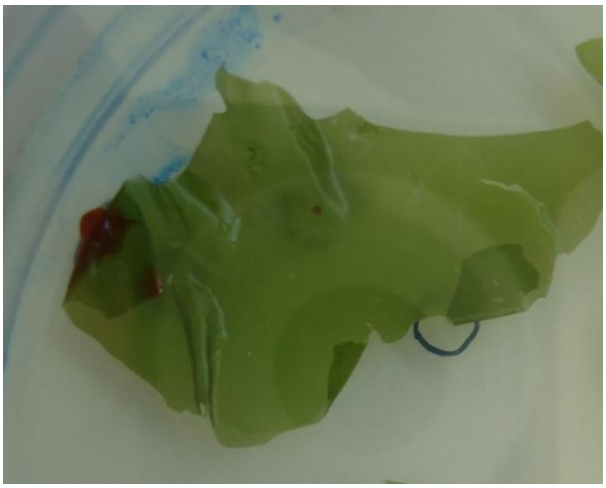


Figure 13 - Portion of Ulva in M13 medium showing the growth of isolates USpP 9 and USpP 10.

4.1.3. Bacterial diversity by Pyrosequencing

Both Autumn and Winter microbial diversity in the samples were analysed by pyrosequencing. These results, although very informative, should be considered cautiously because only one sample from each alga and seawater were analysed.

High-throughput libraries from the raw data contained 1,760,173 sequences. After trimming, QC and USEARCH quality filter pipeline in QIIME a total of 208,806 reads from the samples were retained (Table 4). Good's coverage rarefaction and observed species (OTUs) curves for all individual samples reached a plateau close to 1 (*i.e*. 0.915-0.980) (Fig. 14; Table 4), thus the microbiome of the samples were fully sampled in almost all samples. In some cases a low number of sequences were obtained *(e.g*. SAP, PAP and PWP). In order to improve coverage, these samples were sequenced a second time and the reads were pooled together with the first sequence run. Unfortunately, the PAP sample still yielded a low number of sequences (Table 4) revealing a potential problem.

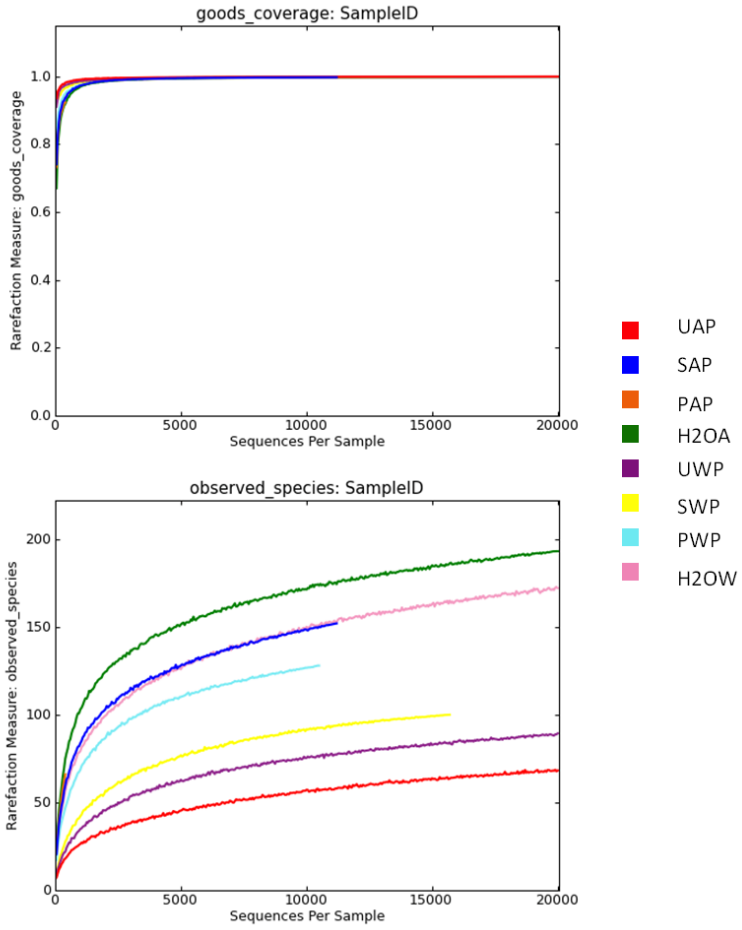


Figure 14 - Good`s coverage rarefaction and observed species curves of the samples.

Table 4 - Good`s coverage estimations per sample.

Samples	Good's coverage	Number of reads
UAP	0.980±0.007	45008
SAP	0.939±0.010	11212
PAP	0.924±0.004	429
H2OA	0.922±0.013	21932
UWP	0.975±0.007	27340
SWP	0.965±0.008	15732
PWP	0.949±0.010	10526
H2OW	0.940±0.011	65668
INT_Ctrl	0.987±0.005	77729
INT_Ctrl	0.986±0.005	27494

The alpha diversity parameters are displayed in Table 5. No significant differences were found among macroalgal species and seawater or seasonality, but interestingly both *Ulva* samples (UAP and UWP) presented the lowest diversity parameters in comparison with the rest of the samples. These values most probably are reflecting the high amount of the "Order *UA01* (Other)" and "Order *Stramenopiles* (Other)" (Fig. 15, Table 6) that probably are chloroplasts from the macroalgae or from the epiphytic microalgae like diatoms (Fig. 4D). In both *Ulva* samples, "Order UA01 (Other)" accounted for 81.8 and 73.9 % of the sequences detected, respectively in Winter and Autumn (Table 6). The "Order *Stramenopiles* (Other)" is represented by 23.0 and 44.0 % of the microbial community in *P. dioica* Winter and *S. muticum* in Autumn, respectively (Table 6). The high proportion of these two groups in the samples mask the real diversity associated with each macroalgae.

Proteobacteria (accounting for 53.4 % of the reads), *Cyanobacteria* (31.4 %), *Planctomycetes* (5.6 %) and *Actinobacteria* (5.5 %) were the most abundant phyla in all the reads (Fig. 15). To a lesser extent reads from *Fusobacteria* (1.5 %) and *Bacteriodetes* (1.5 %), *Firmicutes* (0.9 %) were also observed. Almost exclusive of seawater were SAR406 (0.11 %), OD1 (0.05 %), *Verrucomicrobia* (0.013 %), SBR1093 (0.005 %) and *Chloroflexi* (0.002 %). The percentual importance of the groups changed considerably when the chloroplasts that accounted for k_Bacteria:p_*Cyanobacteria* were excluded and only the bacteria associated to the macroalgae were considered (Fig. 16). *Proteobacteria*, *Actinobacteria*, *Planctomycetes* and *Firmicutes* increased while *Cyanobacteria*, *Bacteroidetes* and *Fusobacteria* decresead (Fig. 16A).

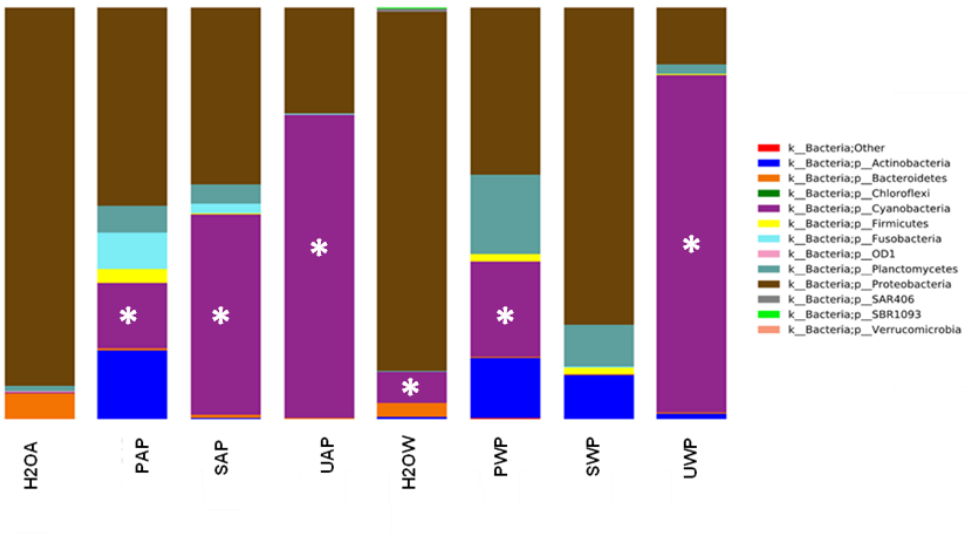


Figure 15 - Proportion of reads of the samples assigned at the phylum level. Data represented are means of the phyla with abundance higher than 0.5%. *Bacteria_Cyanobacteria_Chloroplast

Table 5 - Alpha diversity parameters per sample.

Samples	Chao1	Observed species	Phylogenetic diversity	Shannon
UAP	20.91±5.62	18.25±2.63	1.51±0.41	2.17±0.09
SAP	69.85±7.92	59.02±3.23	3.89±0.26	4.29±0.11
PAP	82.82±4.61	65.92±1.30	5.16±0.05	4.63±0.03
H2OA	87.69±10.32	73.62±4.95	4.69±0.33	4.88±0.11
UWP	26.72±8.77	22.08±2.77	2.61±0.32	1.59±0.11
SWP	33.35±6.63	27.55±3.53	2.59±0.34	2.42±0.12
PWP	57.26±9.78	46.68±3.70	3.96±0.34	3.99±0.10
H2OW	68.11±8.98	56.57±4.32	4.23±0.40	4.38±0.13
INT_Ctrl	25.79±2.66	25.04±1.96	1.25±0.19	3.64±0.07
INT_Ctrl	28.04±3.40	26.9±2.12	1.13±0.18	3.83±0.07

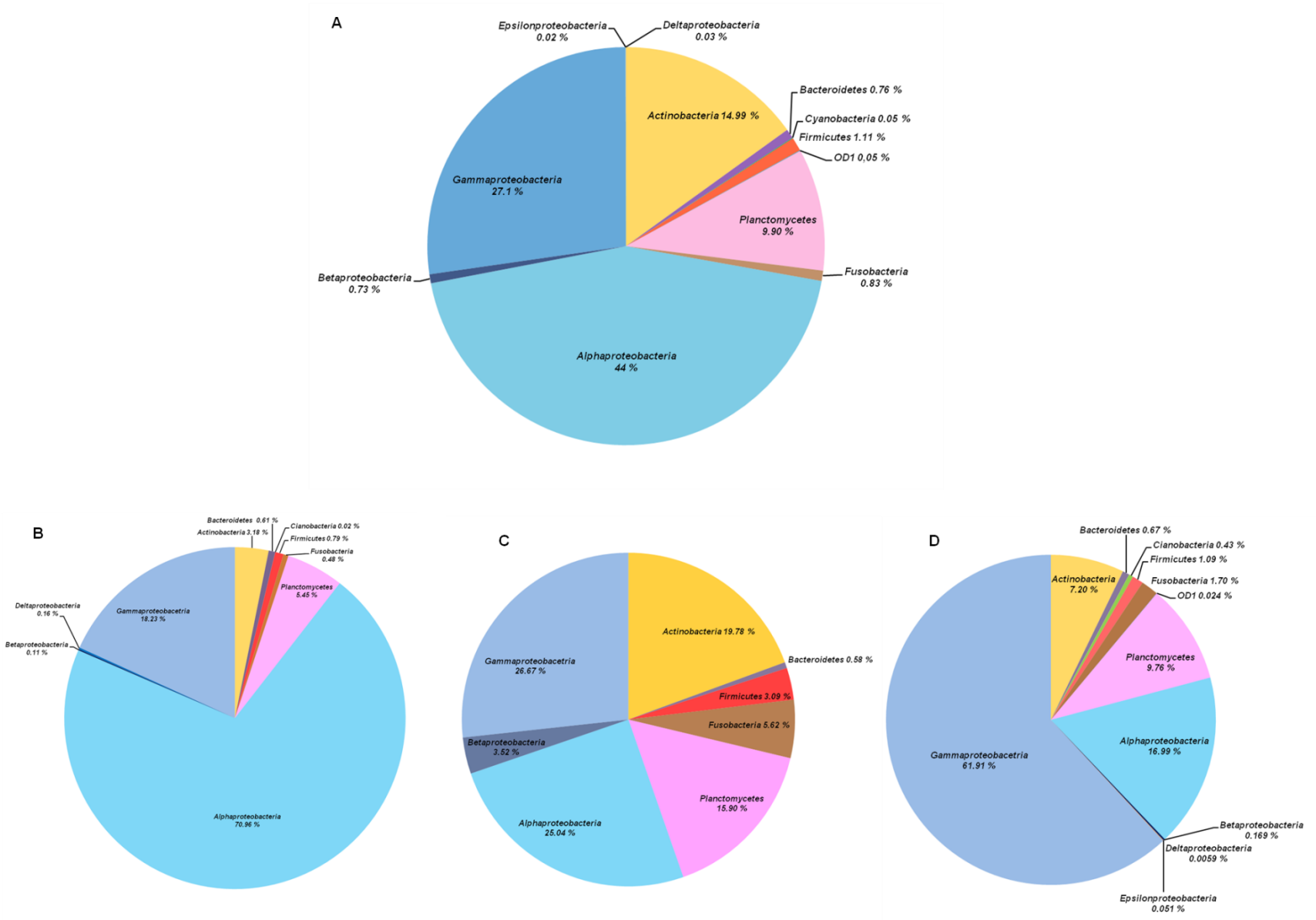


Figure 16 – Bacterial groups obseved in pyrosequencing without the values relative to chloroplast contamination. **A** – Macroalgae and seawater; **B –** *Ulva* sp.; **C –** *P.dioica*; **D –** *S. muticum*.

With exception of *Cyanobacteria*, *Fusobacteria* and *Candidatus* OD1, representatives from the other phyla were also obtained in our isolation study. Other pyrosequencing studies, also dectected *Proteobacteria* (*Alphaproteobacteria* and *Gammaproteobacteria*) as the most abundant groups of bacteria in association with macroalgal biofilms as well as the phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Planctomycetes* (Barott *et al*., 2011; Hollants *et al*., 2013; Miranda *et al*., 2013). These groups are generalistic epiphytes of the different macroalgae and are present in our isolation and pyrosequencing results.

The comparision of bacterial diversity among the three macroalgae is shown in Figure 16 B, C and D. *Ulva* sp. and *S. muticum* presented a higher abundance of *Proteobacteria* (*Alphaproteobacteria* in *Ulva* sp. (77.96 %) and *Gammaproteobacteria* in *S. muticum* (61.91 %)). *P. dioica* had comparatively to the other two macroalgae a higher percentage of *Actinobacteria*, *Planctomycetes*, *Fusobacteria*, *Firmicutes* and *Betaproteobacteria*. *S. muticum* was the only algae that had representatives from the five classes of *Proteobacteria* and also the new *Candidatus* phylum OD1.

Table 6 and Figure 17 show the most abundant genera in the samples. Some bacterial groups were consistently observed in all the macroalgae from the two seasons. Present in all the macroalgae were the *Actinobacteria Propionibacterium* that appears in high percentage in *P. dioica* and *S.muticum* (SWP) and *Corynebacterium* (with exception of PAP) present in low percentage. *Corynebacterium* was previously found associated with macroalgae (Hollants *et al*., 2013).

Cyanobacteria were only detected in Autumn in *Ulva* sp. and *S. muticum*. *Cyanobacteria* are the main autotrophic bacteria associated with algae and appear in high abundance on *Halimeda opuntia* and *Dictyota bartayresiana* (Barott *et al*., 2011). These bacteria have been showed to be related with the nitrogen cycling in algae and in their protection from herbivory (Wilkinson *et al*., 1984; Fong *et al*., 2006). The genus *Acaryochoris* found in this study has been described associated with green, brown and red macroalgae (Ohkubo *et al*., 2006). This bacterium has chlorophyll d which was previously assigned as a product of the red macroalgae *Ahnfeltiopsis flabelliformis* where it lives in an epiphytic association (Murakami *et al*., 2004).

Curiously, the only *Planctomycetes* found in the two seasons associated with all macroalgae were affiliated to the genus *Rhodopirellula*. This result is consistent with our isolations where only *Rhodopirellula baltica* was obtained. However, and although *Rhodopirellula* sp. was abundant in Winter macroalgae, no isolation was obtained. UAP sample showed very low presence of planctomycetes in the pyrosequencing analysis (Fig. 17, Table 6), but curiously, this was the algae that allowed the highest number of

isolates in Autumn. Other planctomycetes such as *Phycisphaerae, Planctomyces* and OM190 were only detected in the surrounding seawater.

P. dioica was the macroalgae that showed the highest diversity of *Firmicutes*. Besides *Staphylococcus*, which appeared in all macroalgae sampled and is known to be associated with these organisms (Hollants *et al*., 2013). Also *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Megasphaera* and *Finegoldia* were found. *Megasphaera* sp. and *Finegoldia* have never been isolated from marine environments.

The genus *Cetobacterium*, representative of the *Fusobacteria* group, was consistently found in the three macroalgae and in a quite high percentage in *P. dioica* from Autumn.

The *Candidatus* phylum OD1 was only identified in *S. muticum* from Autumn as well as in the surrounding seawater.

Of the *Alphaproteobacteria*, *Rhodobactereaceae* namely the genera *Octadecabacter* and *Phaeobacter*, appeared consistently in the three macroalgae and in a good percentage which is consistent with the literature (Hollants *et al*., 2013). The *Pelagibactereaceae* were present in all macroalgae, except in *Ulva* sp. in Winter. These bacteria are known as free-living *Alphaproteobacteria* (Morris *et al*., 2002) and in fact, they were mainly detected in our seawater samples (Table 6). Our results suggest a possible non free-living style of life, not yet described, for this group of bacteria. The family *Erythrobacteraceae* was also present in all macroalgae, namely *Erythrobacter*, result that is consistent with the bacterial isolation achieved in the two seasons. *Sphingobium*, although never reported as a marine genus as far as we know, was present in all macroalgae except in *Ulva* sp. in Autumn.

In the *Betaproteobacteria*, the order *Burkholderiales* with namely *Burkholderia*, *Ralstonia* and the family *Comamonadaceae*, have been found more abundantly in Autumn in *P. dioica*. Association of these bacteria to other macroalgae have been referred (Aires *et al*., 2013; Sweet *et al*., 2013). Furthermore, these macroalgae can be reservoirs of *Burkholderiales* that are pathogens of corals (Sweet *et al*., 2013).

Edwarsiella, *Plesiomonas*, *Enhydrobacter* and *Vibrionaceae* are the *Gammaproteobacteria* present in the three macroalgae from both seasons. The genus *Vibrio* was absent from *P. dioica* in Autumn but one isolated (PAP 46 – Fig. 6) was obtained from this algae. The majority of the *Vibrio* isolates were obtained from *Ulva* sp. (Fig. 6). *Enhydrobacter* was found associated with the green algae *Cladophora glomerata* (Zulkifly *et al*., 2012). Also common in the macroalgae were *Pseudoalteromonas* (except *Ulva* Autumn) and *Pseudomona*s (excepet *Ulva* Winter). Of the *Enterobacteriaceae*, *Serratia* was only observed in *P. dioica* and *S. muticum*, contrary to the *Edwarsiella* and *Plesiomonas*. It is curious the presence of these

enterobacteria, commonly found within animals, in the biofilm of macroalgae. This may be explained by the potential contamination due to the proximity to the sampling place in the beach rocky pool of sewage. *Reinekea*, an *Oceanospirillales*, was present in high abundance in *P. dioca* in Winter but absent in Autumn. This genus was found associated with marine sediments (Romanenko *et al*., 2004) and seawater from the Mediterranean Sea (Pinhassi *et al*., 2007). The genus *Alteromonas* was only observed in the seawater although isolates from *Ulva* sp. and *S. muticum* were obtained. The presence of the *Candidatus* Portiera in the seawater as well as in macroalgae is somehow strange as this bacterium is an obligate endosymbiont in the whiteflys (Jiang *et al*., 2012). *Cocleimonas* was present in the three macroalgae and in high abundance in *Ulva* sp. in Winter. This genus is described in other studies associated with red algae biofilm (Miranda *et al*., 2013; Wu *et al*., 2014).

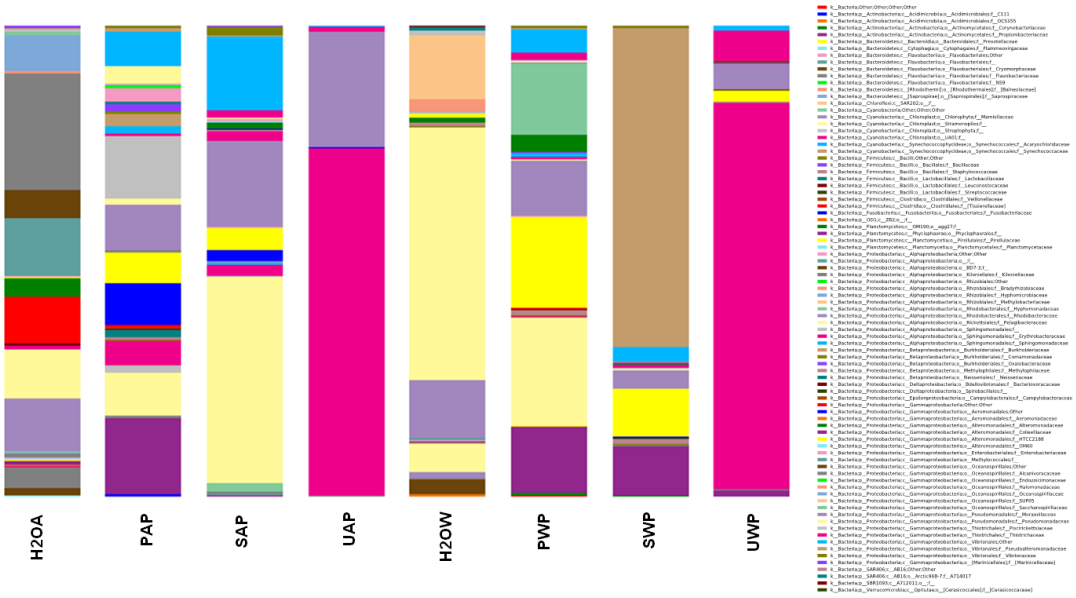


Figure 17 - Proportion of reads of the samples assigned at the genera level.

Table 6 - Abundance of the OTUs found in the samples. Genera level identification is presented where possible. Data represented are relative abundance (%).

Phylum/Class	OTU	H2OA	PAP	SAP	UAP	H2OW	PWP	SWP	UWP	INT_Ctrl	INT_Ctrl
Actinobacteria	*Propionibacterium*	0	16.1	0.1	0.02	0.02	14.0	10.5	1.4	0	0
	Corynebacterium	0	0	0.02	0.004	0	0.7	0.27	0.01	0	0
	Acidimicrobiales C111	0.09	0.47	0.45	0	0.002	0	0	0	0	0
	Acidimicrobiales OCS155	0	0	0	0	0.4	0	0	0	0	0
Cyanobacteria	SAR202	0	0	0	0	0.13	0	0	0	0	0
	Order UA01 (Other)	0.2	5.4	2.4	73.9	0.08	0.2	0.06	81.8	0	0
	Order *Stramenopiles* (Other)	0.05	9.1	44.0	0.05	5.9	23.0	0.01	0.1	0	0
	Mamiellaceae	0	0	0	0	1.3	0	0	0	0	0
	Acaryochloris	0	0	0.7	0.007	0	0	0	0	0	0
	Synechococcus	0	0	0	0	0.03	0	0	0	0	0
	Order *Streptophyta*	0.005	1.4	0.03	0	0	0.05	0.04	0.02	0	0
Firmicutes	*Lactobacillus*	0	1.4	0	0	0	0	0.1	0	15.3	16.9
	Pediococcus	0	0.2	0	0	0	0	0	0	6.0	7.5
	Streptococcus	0	0	0	0	0.002	0.1	0.2	0.007	0	0
	Leuconostoc	0	0.23	0.009	0	0	0.05	0	0.007	0	0
	Staphylococcus	0	0.47	0.04	0.007	0.008	1.2	1.1	0.2	0	0
	Bacillus	0	0	0.009	0	0	0	0	0.01	18.4	16.2
	Family *Bacillaceae* (Other)	0	0	0	0	0	0	0	0.01	3.5	3.9
	Megasphaera	0	0.2	0	0	0	0.06	0.03	0	0	0
	Finegoldia	0	0.47	0	0	0	0.08	0.03	0	0	0
Bacteroidetes	*Prevotella*	0	0	0	0	0.002	0.2	0.006	0	0	0
	Crocinitomix	0.2	0	0	0	0.002	0	0	0	0	0
	Owenweeksia	1.5	0	0	0	0	0	0	0	0	0
	Cellulophaga	2.7	0	0.01	0	0.002	0	0	0.004	0	0
	Flavobacterium	0.08	0.2	0.01	0	0.01	0	0.01	0	0	0
	Kordia	0.005	0	0.03	0	0	0	0.02	0	0	0
	Krokinobacter	0.4	0.5	0.2	0.05	0.002	0.04	0.03	0	0	0
	Maribacter	0.5	0	0.2	0	0.002	0	0	0	0	0
	Persicivirga	0.2	0	0	0	0.002	0	0.01	0.004	0	0
	Balneola	0.1	0	0.02	0	0	0	0	0	0	0
	Saprospira	0	0	0	0	0.003	0	0	0.05	0	0
	Family *Flammeovirgaceae* (Other)	0.06	0	0	0	0	0	0	0	0	0
	Family *Cryomorphaceae* (Other)	0	0	0	0	3.3	0	0	0	0	0
	Family *Flavobacteriaceae* (Other)	0.3	0	0.03	0.06	0	0	0	0.07	0	0
	Family NS9	0	0	0	0	0.1	0	0	0	0	0
	Order *Flavobacteriales*(Other)	0	0	0.2	0	0.005	0.03	0.02	0.03	0	0
Fusobacterium	*Propionigenium*	0	0	0.4	0	0	0	0	0	0	0
	Cetobacterium	0	8.9	2.0	0	0	0.2	0.1	0.1	0	0.4
	Psychrilyobacter	0	0	0.03	0	0.01	0.02	0	0.004	0	0
Planctomycetes	*Rhodopirellula*	0.2	6.5	4.7	0.09	0.2	19.1	10.1	2.3	0	0
	Phycisphaerales	0.8	0	0	0	0.03	0	0	0	0	0
	Planctomyces	0.4	0	0	0	0	0	0	0	0	0
Alphaproteobacteria	*Octadecabacter*	1.1	5.8	7.0	1.3	7.1	5.9	1.6	1.4	0	0
	Phaeobacter	2.5	0	0.5	0.4	0.1	0.2	0.01	0.2	0	0
	Thalassospira	0.7	0.5	0	0	0	0	0	0	0	0
	Devosia	0,2	0	0	0	0	0	0	0	0	0
	Hyphomonas	0.3	0	0.009	0	0	0	0	0	0	0
	Jannaschia	0.05	0	0	0	0	0.03	0.3	0	0	0

	Marivita	0.01	0	0	0.002	0	0	0	0	0	0
	Erythrobacter	0.2	0	0.4	0.07	0	0.3	0.02	0	0	0
	Sphingobium	0	1.2	0.08	0	0.002	0.5	0.1	0.07	0	0
	Family *Pelagibacteraceae* (Other)	10.3	1.2	0.009	0.004	53.7	0.2	0.3	0	0	0
	Order *Rhizobiales* (Other)	0.02	0	0	0	0	0.2	0	0	0	0
	Order *Sphingomonadales* (Other)	0.01	13.3	0	0	0	0	0.04	0	0	0
	Order *BD7-3*	0.06	0	0.1	0.02	0.008	0.06	0	0.1	0	0
	Family *Sphingomonadaceae* (Other)	0.005	0.2	0.02	0	0.003	0.04	0	0.004	0	0
	Family *Erythrobacteraceae* (Other)	0.4	0.7	1.1	0.2	0.002	0.1	0.6	0.2	0	0
	Family *Methylobacteriaceae* (Other)	0	0	0.02	0	0.002	0.01	0	0.04	0	0
	Family Bradyrhizobiaceae (Other)	0.01	0.2	0.009	0	0	0	0.1	0.03	0	0
	Family *Rhodobacteraceae* (Other)	7.7	3.3	8.3	3.5	5.2	4.6	2.1	2.4	0	0
	Family *Rhodobacteraceae* (Other)	0.01	0.5	2.2	19.2	0.01	1.0	0	1.5	0	0
Gammaproteobacteria	*Cocleimonas*	0.01	0	1.5	0.002	0.01	1.8	0	6.5	0	0
	Pseudoalteromonas	0.2	0.9	0.2	0	0.1	0.1	67.7	0.004	0	0
	Pseudomonas	0.1	3.5	0.1	0.004	0	0.3	0.1	0	17.6	16.8
	Reinekea	0.9	0	0.1	0.002	0.01	14.8	0	0	0	0
	Alcanivorax	24.8	0	0	0	0.1	0	0	0	0	0
	Agarivorans	0.3	0	0	0	0	0	0.04	0	0	0
	Alteromonas	0.7	0	0	0	0.01	0	0	0	0	0
	Candidatus Endobugula	0.01	0	0.3	0	0.002	0.1	0	0.004	0	0
	Candidatus Portiera	0.01	0	0.01	0	2.8	0.02	0	0.007	0	0
	Halomonas	0.3	0	0	0	0.002	0	0	0	0	0
	Glaciecola	0.02	0	0.1	0.01	0.05	0.4	0	0.1	0	0
	HTCC2207	0	0	0	0	1.0	0	0	0	0	0
	HTCC	0.4	0	0.01	0	0.8	0	0.04	0	0	0
	Marinobacter	1.8	0	0	0	0	0.1	0	0	0	0
	Thalassomonas	0.01	0	0	0	0.04	0	0	0	0	0
	Edwardsiella	0	0.7	0.3	0.007	0	0.01	0.01	0.007	0	0
	Plesiomonas	0.01	0.9	0.2	0.01	0.002	0.03	0.02	0.01	0	0
	Serratia	0	1.2	0.04	0	0	0.04	0.01	0	0	0
	Oceaniserpentilla	0.005	0	0	0	0	0	0	0	0	0
	Oleispira	0.01	0	0	0	0.1	0.03	0.03	0	0	0
	Acinetobacter	0	0	0	0	0	0.2	0.03	0	0	0
	Enhydrobacter	0	0.2	0.06	0.02	0	0.05	0.006	0.05	0	0
	Photobacterium	0	0.2	0.2	0.004	0.005	0	0.05	0	0	0
	Vibrio	0	0	0.6	0.01	0.003	0.09	0.1	0.02	0	00
	Marinicella	0.5	0	0.1	0.002	0.002	0	0	0	0	0
	Order *Vibrionales* (Other)	0.1	7.2	15.8	0.2	0.02	5.0	3.2	0.9	14.0	13.4
	Class *Gammaproteobacteria* (Other)	9.9	0	0	0	0	0	0.2	0	2.2	2.2
	Family *Enterobacteriaceae* (Other)	0	0	0.03	0	0	0.01	0	0	17.2	18.2
	Family *Aeromonadaceae* (Other)	0	0	0	0.002	0.003	0	0.03	0	0	0
	Family *Alteromonadaceae* (Other)	1.0	0	1.0	0	0.005	3.1	0.03	0	0	0
	Family *Colwelliaceae* (Other)	0	0	0.05	0	0.006	0	0	0	0	0
	Family *HTCC2188* (Other)	0.02	0	0	0	0.008	0	0	0	0	0
	Family *OM60* (Other)	0.04	0	0.1	0	0.1	0.01	0.02	0	0	0
	Order *Aeromonadales* (Other)	0.005	0	0.02	0	0.003	0.02	0.006	0	0	0
	Order *Methylococcales* (Other)	12.4	0	0.01	0	0.003	0	0	0	0	0
	Order *Oceanospirillales* (Other)	6.0	0	0	0	0.1	0	0	0	0	0
	Family *Endozoicimonaceae* (Other)	0	0.5	0	0	0	0	0	0	0	0
	Family *Oceanospirillaceae* (Other)	7.5	0.2	0	0	0.01	0	0	0	0	0

	Family *Saccharospirillaceae* (Other)	0.005	0	0.03	0	0.002	0.4	0	0	0	0
	Family *Vibrionaceae* (Other)	0.005	0.2	1.2	0.02	0	0.5	0.3	0.05	0	0
	Family *Piscirickettsiaceae*	0.3	0	0.01	0.002	1.0	0.01	0.03	0	0	0
	Family *SUP05* (Other)	0.01	0.2	0	0	13.4	0	0	0.004	0	0
Betaproteobacteria	*Burkholderia*	0	2.8	0.07	0	0	0.06	0	0.004	0	0
	Ralstonia	0	1.6	0.07	0	0	0.1	0	0	0	0
	Neisseria	0	0.5	0	0	0	0.04	0.05	0.007	0	0
	Family *Comamonadaceae* (Other)	0	0.5	0.05	0	0.008	0.07	0	0.03	0	0
	Family *Methylophilaceae*	0.005	0	0.009	0	0.8	0	0	0.004	0	0
Deltaproteobacteria	*Bacteriovorax*	0.6	0	0.009	0	0	0	0	0	0	0
	Order *Spirobacillales*	0	0	0	0.04	0	0	0	0.04	0	0
Epsilonproteobacteria	*Arcobacter*	0	0	0.03	0	0.04	0	0	0	0	0
	Campylobacter	0	0	0	0	0	0	0.05	0	0	0
Verrucomicrobia	Family *Cerasicoccaceae*	0.005	0	0	0	0.1	0	0	0	0	0

4.2. Analysis of bacterial seasonal variation

The physical parameters measured in the study area in the four seasons are represented in Table 7. The most striking difference was obtained in Winter where the water temperature was much lower than in the other sampling times. Higher values of pH were obtained in Spring and Summer.

Table 7 - Parameters analyzed from seawater.

	October 2014 **Autumn**	**January 2015** **Winter**	**May 2015** **Spring**	**July 2015** **Summer**
Temperature (TºC)	21 ºC	10 ºC	20 ºC	20 ºC
Salinity	34.2 ppt	33.3 ppt	35.6 ppt	34.2 ppt
Conductivity	51.9 mS/cm	50.8 mS/cm	53.7 mS/cm	51.9 mS/cm
pH	8.00	8.07	9.07	8.70

The seasonal variation in the bacterial communities was studied by two molecular methods: Pyrosequencing in samples from two seasons (Autumn and Winter) and DGGE fingerprinting in one-year sampling. The three macroalgae *Ulva* sp., P*. dioica* and *S muticum* were compared among them and with the surrounding seawater.

For the 16S rRNA gene DGGE profiles, three different gradient concentrations were tested and the gradient 40 to 70% was the one where the band profiles were clearer and better band separation achieved (Fig. 18). For the DGGE gel analyses relative quantity matrices were created and used to produce a dendrogram (Fig. 19 A), and nMDS plots (Fig. 19 B). Two samples from Spring (USpP 1 – lane 15 and SSpP 1-lane 19) showed low quality for analysis due to low DNA concentration that may be interfering with the analysis.

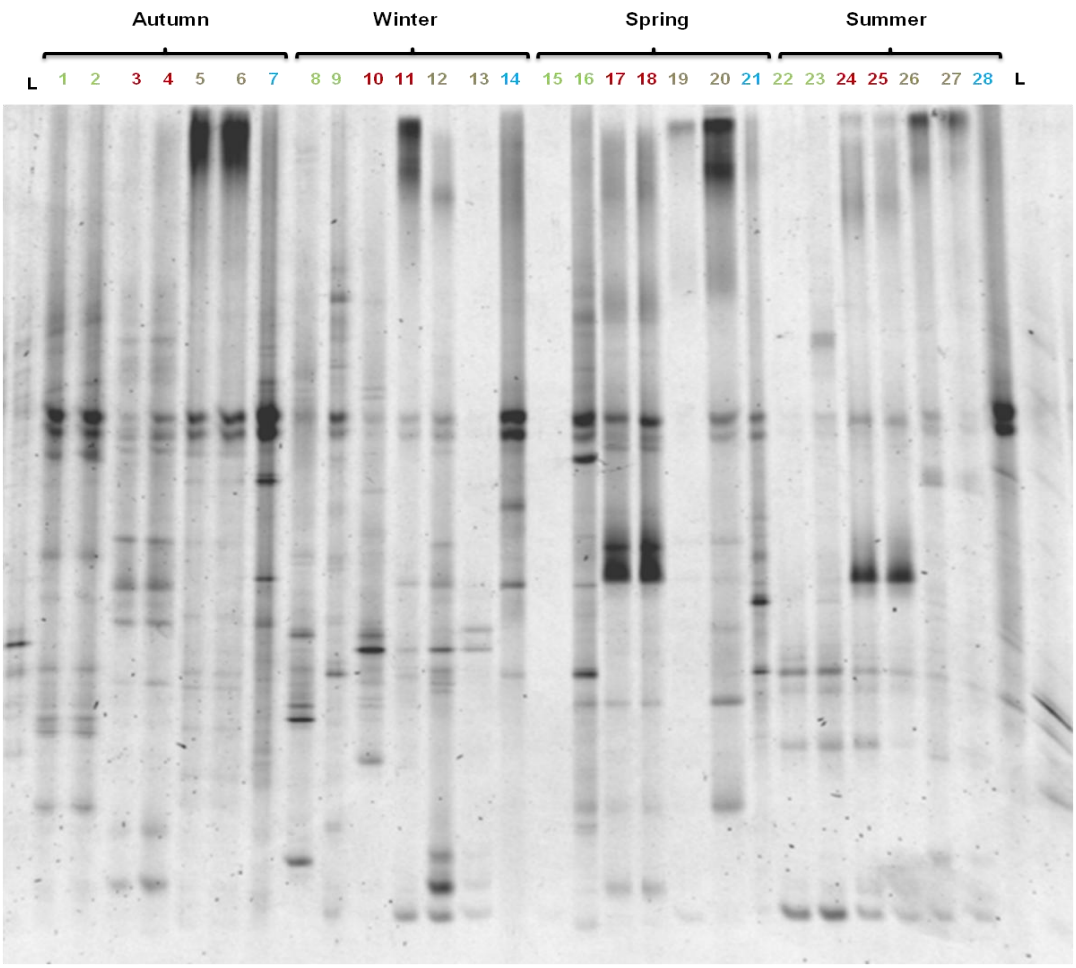


Figure 18 - Seasonal comparison of bacterial communities of *Ulva* sp. (lanes 1, 2, 8, 9, 15, 16, 22, 23), *P. dioica* (lane 3, 4, 10, 11, 17, 18, 24, 25), *S. muticum* (5, 6, 12, 13, 19, 20, 26, 27) and seawater (7, 14, 21, 28) through the analysis of 16S rRNA gene DGGE gel profiles. L – Ladder.

No significant differences ($p > 0.05$ in the one-way ANOSIM) between all the macroalgae and seawater were observed. These results are evidenced by the absence of significative clustering in the dendogram or in the nMDS (Figs 19 A and B). However, *Ulva* from different seasons (Autumn, Winter and Spring), *Porphyra* from Spring and Summer and seawater from Autumn, Winter and Summer showed closest proximity. In general, duplicates of each sample are clustered together showing the uniformity of their bacterial community. The highest inter-individual similarities were obtained for the three Autumn algae (≥ 88 %) and the lowest in the Winter algae (54 – 64 %) (excluding *Ulva* sp. and *S. muticum* Spring samples). As already referred, no significant differences were obtained in the pyrosequencing analysis of the alfa-diversity. Both methods are, thus, in agreement regarding absence of statistical significant difference among the bacterial communities of macroalgae and surrounding seawater. However, a clear difference between the biofilm communities of macroalgae and surrounding seawater is commonly observed (Bengtsson *et al*., 2010; Burke *et al*., 2011b; Goecke *et al*., 2013). Furthermore, it has also been described a macroalgal

host-specific bacterial community as observed by Lachnit *et al*., (2011) for *Fucus vesiculosus*, *Gracilaria vermiculophylla* and *Ulva intestinalis*.

Autumn was the season with the lowest variation of bacterial community among the three macroalgae and the seawater (Fig. 20 C and D). Tujula *et al*., (2010) in their study of *Ulva australis* different specimens also observed that the Autumn was the season with lowest variation between the communities of individual hosts.

The stress value obtained in the nMDS is between 0.2 and 0.3 (0.257), which means that this 2D plot (Fig. 19B) is a quit good representation of the real differences among samples (Clarke, 1993).

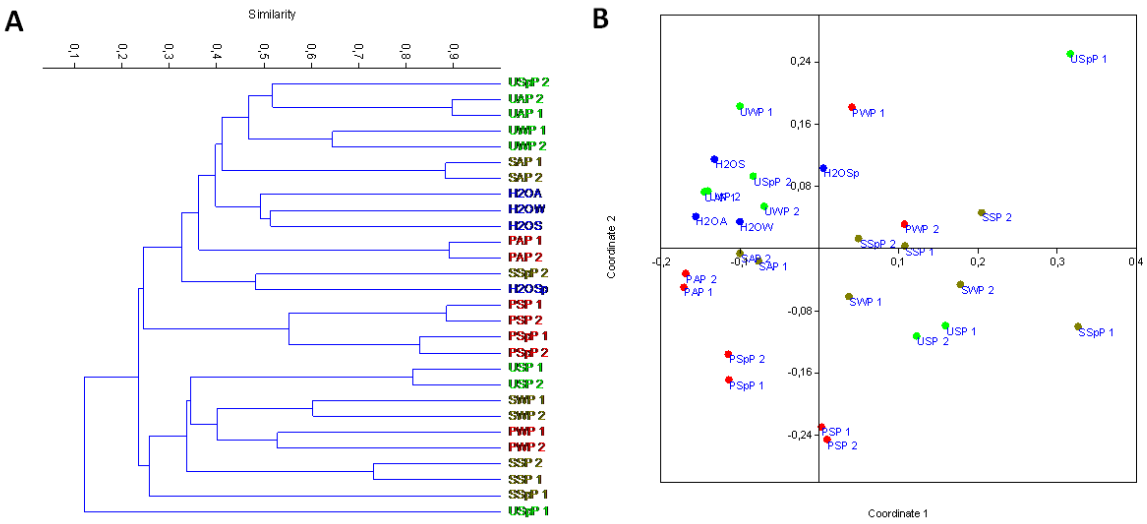


Figure 19 - (A) Dendrogram of DGGE profiles of macroalgae and seawater samples, based on Bray-Curtis similarity. **(B)** Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.257) based on Bray-Curtis similarity.

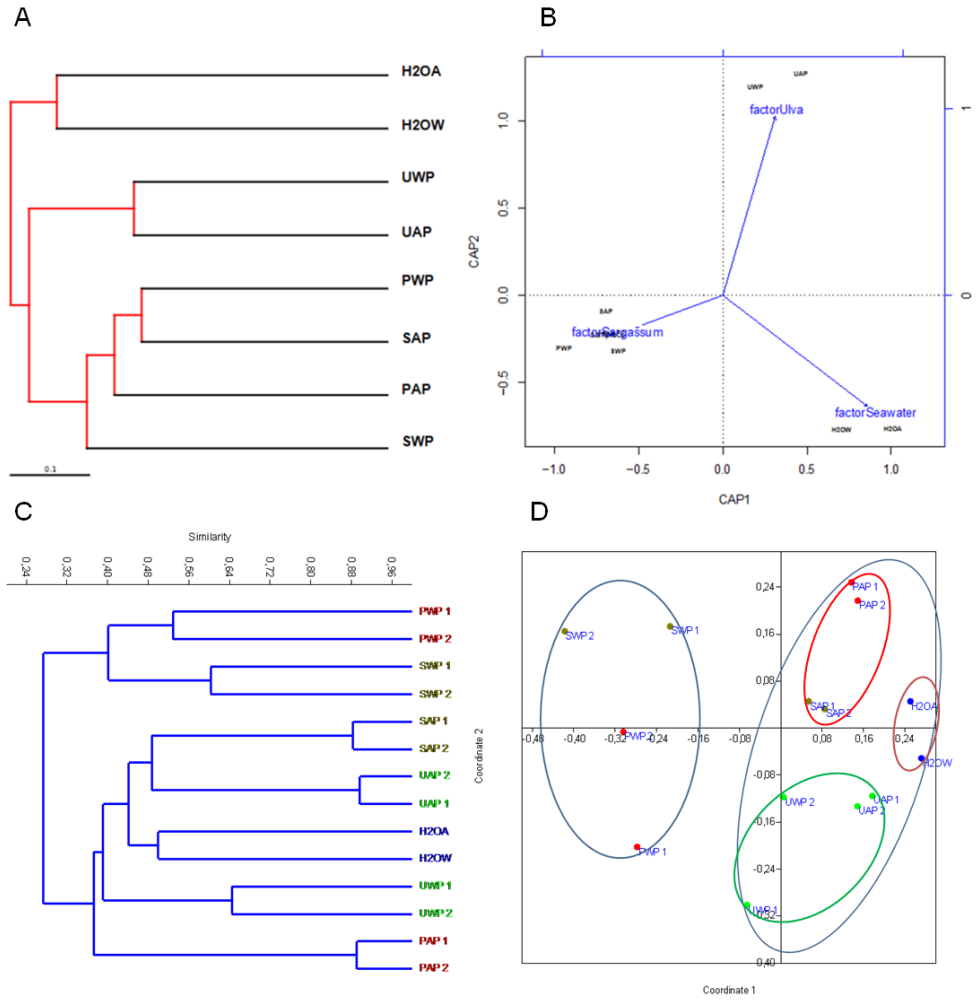


Figure 20 - (A) Dendogram obtained by pyrosequencing based on Bray-Curtis similarity. **(B)** Distance-based ReDundancy Analysis (dbRDA) of beta rarefaction metrics of the samples constrained by species and seawater: Bray-Curtis dissimilarity. **(C)** Dendrogram of DGGE profile of macroalgae and seawater samples in Autumn and Winter, based on Bray-Curtis similarity. **(D)** Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.1645) based on Bray-Curtis similarity.

In the pyrosequencing analysis, the seawater samples are separated in a different branch from the macroalgae (Fig. 20 A and B) and at a lower level the *Ulva* sample. Mixed together are the *S. muticum* and *P. dioica* samples revealing higher similarity of their microbial communities. Even thought no significant differences were obtained among the various bacterial communities of the macroalgae and seawater, figure 20B shows that the communities of *S. muticum* and *P. dioica* are much closer than that of *Ulva* or of the seawater. This is also preceptivel in the DGGE analysis (Fig. 20 C and D) were *P. dioica* and *S. muticum* from Autumn and *P. dioica* and *S. muticum* from Winter are more closely together.

Table 9 shows the ANOSIM analysis (Bray-Curtis) of the DGGE profiles obtained when considering each macroalgae and the seawater. This allows based on R values to perform parwise comparision of bacterial communities among macroalgae

and seawater. R varies between –1 and +1, where 1 corresponds to complete separation of the compared groups and 0 indicates no separation (R ≥ 0.75 means the complete separation of groups, R = 0.5 means clear separation but overlapping of groups, R = 0.25 means no separation of groups) (Clarke and Warwick, 2001). In the pairwise comparisons, R values ranged from 0.03 to 0.44 which indicates that all macroalgae biofilm and planctonic bacteria are similar. Two of the values were not supported statistically (*Ulva* sp./*P. dioica* and *Ulva* sp./H2O). This analisys reinforces the previous ones already referred.

Table 8 – R and p values obtained in ANOSIM (Bray-Curtis measure) based on the DGGE profiles when macroalgae and seawater were constrained.

R ─ p values	***Ulva* sp.**	***S. muticum***	***P. dioica***
***Ulva* sp.**	─	─	─
S. muticum	0.34 ─ 0.005	─	─
P. dioica	0.15 ─ 0.079	0.29 ─ 0.011	─
Seawater	0.03 ─ 0.360	0.44 ─ 0.010	0.27 ─ 0.050

Both methods, DGGE fingerprinting and pyrosequencing, evidenced quite similar levels of diversity between Autumn and Winter samples (Tables 10 and 11). The lower value obtained in Winter is most probably due to the much lower temperature of seawater (Table 7). In the pyrosequencing analysis without the exclusion of "chloroplasts" percentages, *Ulva* diversity was the lowest obtained contrary to what was observed in the DGGE analysis. This result supports the interference due to the high levels of chloroplasts in the macroalgal samples.

Table 9 - Alpha diversity parameters by species, seawater and seasonality in pyrosequencing analysis.

Species/Seasonality	**Chao1**	**Observed species**	**Phylogenetic diversity**	**Shannon**
Ulva sp.	34.30±5.02	20.17±1.92	2.06±0.55	1.88±0.29
P. dioica	93.24±15.05	56.30±9.62	4.56±0.60	4.31±0.32
S. muticum	67.84±20.18	43.29±15.74	3.24±0.65	3.36±0.94
Seawater	97.78±9.74	65.10±8.53	4.46±0.23	4.63±0.25
Winter	72.03±25.20	43.69±16.62	3.70±0.97	3.34±1.13
Autumn	89.04±31.03	57.65±20.34	4.15±1.42	4.09±0.98

Table 10 – Alpha diversity parameters by species, seawater and seasonality in DGGE analysis.

Species/Seasonality	Chao1	Shannon
Ulva sp.	22.75±0.96	2.93±0.08
P. dioica	19.75±2.87	2.86±0.15
S. muticum	16.75±3.95	2.61±0.16
Seawater	19.50±7.78	2.70±0.40
Winter	17.57±4.58	2.69±0.24
Autumn	21.86±1.68	2.88±0.11

4.3. Search of potential PKS-I and NRPS genes

All bacteria isolated from macroalgae were screened for the presence of potential PKS-I and NRPS genes. The products of the PCR reaction were assessed by gel electrophoresis for the presence of approximately 700 bp and 1000 bp size amplicons (Fig. 22), specific for PKS-I and NRPS genes respectively (Neilan *et al*., 1999; Kim *et al*., 2005).

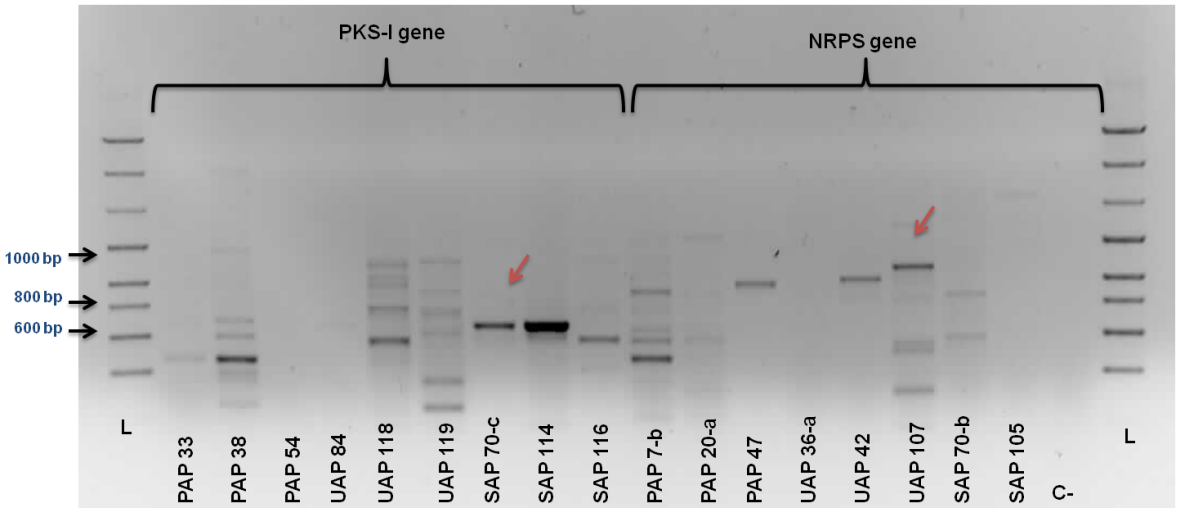


Figure 21 - Example of an electrophoresis gel of PKS-I and NRPS amplifications (arrow) in some strains representative of all samples. L – Ladder (GeneRuler^{TM} DNA Ladder Mix).

The levels of PKS-I gene were higher than the ones of NRPS gene. Comparable levels of PKS-I gene were obtained in bacteria from the three macroalgae (Fig. 23). In the case of the NRPS gene, bacteria from *P. dioica* and *Ulva* sp. revealed a comparable abundance of this gene. However, a smaller percentage was obtained for *S. muticum* bacteria (Fig. 23).

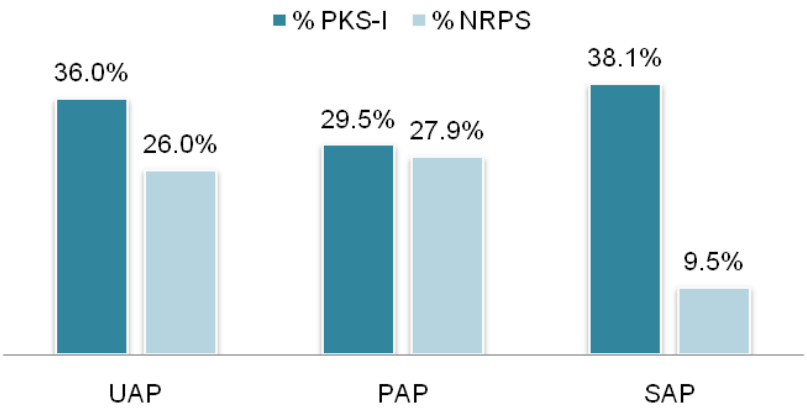


Figure 22 - Percentage of potential PKS-I and NRPS genes search in the bacterial isolated from each macroalgae.

Bioactive potential of bacterial isolated from macroalgae was also observed by Wiese *et al*., (2009) and Penesyan *et al*., (2009).

Genera that possessed only one and both potential genes were *Vibrio*, *Rhodopirellula* and *Loktanella* (Fig. 6) which strongly suggest possible bioactive capacity. The family *Vibrionaceae* is well known for their ability to produce bioactive secondary metabolites which are mainly non-ribosomal peptides (Mansson *et al*., 2011). Besides NRPS several PKS-I genes were also observed in this study as also observed by Graça *et al*. (2013). The antibiotic Andrimid is produced by *Vibrio* spp. using a hybrid NRPS-PKS system as was referred by Mizuno *et al*. (2013). The same antibiotic is also produced by *Vibrio coralliilyticus* which production is stimulated by chitin (Wietz *et al*., 2011). Machado *et al*., (2015) in his study with antiSMASH software, demonstrated the great potential of *Vibrio* for secondary metabolite production.

Rhodopirellula baltica is known to possess two small nonribosomal peptide synthetases (NRPSs), two monomeric polyketide synthases (PKSs) and a bimodular hybrid NRPS–PKS (Donadio *et al*., 2007). Further secondary metabolites genes were observed in 13 planctomycetes genomes by Jeske *et al*. (2013). These genes included bacteriocin encoding genes, putative lantibiotic-encoding gene, ectoine synthesis gene cluster and putative phenazine encoding gene cluster. Preliminary unpublished results obtained in our laboratory, already evidenced presence of PKS-I and NRPS genes in planctomycetes as well as antimicrobial activity.

Loktanella has not been found to possess bioactivity nor secondary metabolism related genes (Machado *et al*., 2015), results that are contrary to the ones obtained in this study.

PKS-I genes were also found in the *Proteobacteria*: *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Halomonas*, *Pseudomonas,* *Stappia* and

Labrenzia; in the *Actinobacteria*: *Brevibacterium* and *Microbacterium*; in the *Firmicutes*: *Salinococcus* and in the *Bacteroidetes*: *Granulosicoccus*, *Krokinobacter* and *Zobellia*.

NRPS genes were also found in the *Proteobacteria*: *Alteromonas* and *Pseudoalteromonas;* in the *Bacteroidetes: Maribacter, Dokdonia, Tenacibaculum* and *Zobellia;* in the *Firmicutes: Staphylococcus*.

4.4. Quorum sensing studies

Perceive microbial biofilms, where a great number of bacterial interactions happen, it would be relevant to get insights about the communication between bacteria key players in the intricate structure of the biofilm. For this reason, communication was addressed by the study of quorum sensing, namely by the analysis of the *luxS* gene expression. Our study was based on Bodor *et al*. (2008) work. Several amplification trials of the *luxS* gene were assayed. However, no amplification was ever obtained. Besides the isolated strains from the macroalgae, amplification with *Vibrio* sp., *Pseudoalteromonas* sp. and *Halomonas* sp. were assayed and used as controls in the experiment. These strains as well were unable to express *luxS* gene.

This work could have given us information on specific bacteria that are fundamental in communication and biofilm formation and development.

5. Conclusion

Rocky tidal pools are inhabited by a diverse community of macroalgae that share a quite closed environment. Comparative study of the bacterial communities in macroalgae and surrounding seawater in such a closed environment as far as we know has not been done.

Confirmation of a varied microbial biofilm in the three macroalgae studied showing the intimate bacteria-macroalgae ecological relationship was obtained by optical and scanning electron microscopy.

Both culture-dependent and independent methods confirmed the presence of the genera *Rhodopirellula*, *Erythrobacter*, *Pseudomonas*, *Pseudoalteromonas* and *Vibrio* in the macroalgae samples, but each method also allowed to identify other different genera. In both methods *Proteobacteria* was the dominant group, result consistent with what has been described in the literature but *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Firmicutes*, *Fusobacteria* and *Cyanobacteria* were also found. It was confirmed that *R. baltica* is a common inhabitant of the epibacterial community of macroalagae.

The seasonal study of the bacterial community by DGGE fingerprinting and pyrosequencing analyses showed a great similarity in the diversity found among the macroalgae and the surrounding seawater. Nor the seasons nor the macroalgal species had a determinant influence on bacterial community. It seems that the factor “proximity” was fundamental in the uniformity of the bacterial diversity obtained.

The molecular analysis of secondary metabolite genes evidenced the great potential of bacteria isolated from macroalgae. The complexity present in macroalgae biofilms where bacteria have to compete against other microorganisms favours the selection of species with high bioactive potential. Furthemore, bacterial communication is also fundamental in this microenvironment. Our attempts to study quorum sensing signalling through the analysis of the *luxS* gene expression were not achieved.

6. Future perspectives

The work presented in this thesis is a relevant contribution in the study of the epibacterial diversity found in macroalgae surface opening future perspectives.

Confirmation of the bacterial communities by pyrosequencing should be confirmed by the analysis of replicates. Improvement of the DGGE fingerprinting technique as well as sequencing of the bands obtained would allow complementary information of the biodiversity present in the macroalgae and seawater. These would also allow the phylogenetic identification of bacteria from Spring and Summer. Also the phylogenetic identification of all the isolates should be completed. Another interesting study would be the analysis of other macroalgae or other organisms harbouring bacterial community in the same environment. This overall approach would allow the confirmation of the similarity of the diversity shared by the different macroalgae and the seawater in such a confined microhabitat which is the rocky pool where the samples were collected.

As the bacterial isolates revealed to possess genes associated with the production of bioactive compounds it is important to continue the screenning of this potential through realization of bioactive assays. The amplicons of the potential PKS-I and NRPS genes obtained should be sent for sequencing for a precise confirmation of their nature. Moreover, the molecular approach could be enlarged by the analysis of other genes know to be envolved in the production of secondary metabolites.

To complement our insights into the biofilm of macroalgae, further work and approaches should be attempt to elucidate the mechanisms and bacteria responsible for the quorum sensing in this microenvironment.

This multidisciplinary study would provide further information on the understanding of the diversity and function of a marine ecosystem still understudied. The publication of the manuscript in a peer review journal is envisaged.

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