Universidade de Aveiro Departamento de Química
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Patrícia Maia Domingues

Isolamento de Bactérias Estuarinas Produtoras de Biossurfactantes

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Isolation of Estuarine Biosurfactant-Producing Bacteria

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Professora Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor Newton Carlos Marcial Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

Dedico este trabalho aos meus pais que sempre me apoiaram

o júri

presidente

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palavras-chave bactérias hidrocarbonoclásticas, biossurfactante, biorremediação, hidrocarbonetos do petróleo, ria de Aveiro

resumo

A biorremediação é tida como uma possível estratégia na recuperação de ecossistemas contaminados com hidrocarbonetos. A aplicação eficaz desta tecnologia é, no entanto, muitas vezes limitada pela natureza hidrofóbica dos contaminantes. O recurso a estirpes bacterianas simultaneamente degradadoras de hidrocarbonetos e produtoras de biossurfactantes apresenta um enorme potencial na reciclagem de compostos hidrofóbicos. Assim, o objectivo deste trabalho consistiu em avaliar o potencial biotecnológico do sistema estuarino da Ria de Aveiro quanto à presença de bactérias hidrocarbonoclásticas produtoras de biossurfactantes e a avaliação de várias combinações de inóculos ambientais e fontes de carbono para a obtenção de isolados bacterianos de interesse.

Para tal foram realizadas experiências em meios selectivos (diesel, crude e parafina) a partir de inóculos de diferentes matrizes ambientais: amostras da microcamada superficial (SML), sedimentos estuarinos e rizosfera de bancos de *Halimione portulacoides*, uma planta halófita dos sapais da Ria de Aveiro. O desenvolvimento da cultura ao longo do período de incubação foi avaliado pela contagem de unidades formadoras de colónias (CFUs). A cultura selectiva com maior teor de bactérias cultiváveis foi a de crude-sedimento e aquela em que a abundância bacteriana foi mais baixa foi a de diesel-rizosfera. A partir da análise dos perfis de DGGE dos fragmentos do gene 16s rRNA do DNA total extraído das culturas selectivas verificou-se que no fim do período de incubação, o grau de semelhança entre as comunidades bacterianas das culturas selectivas é relativamente baixo. Pelo índice de diversidade de Shannon-Weaver a maior diversidade estrutural das comunidades bacterianas encontra-se nas culturas selectivas de parafina (2,5231), seguidas das de crude (2.2509) e das de diesel (1.6727). Das culturas selectivas, foi obtido um conjunto de isolados que foi testado quanto à capacidade de produção de biossurfactantes pelo método *atomized oil*. De 66 isolados testados, 17 produziram resultado positivo (25,8%), sendo a água da SML a matriz ambiental com melhores resultados e o diesel a melhor fonte de carbono para o isolamento de bactérias produtoras de biossurfactantes.

Tendo em conta o elevado número de isolados obtidos e a percentagem de produtores de biossurfactantes, pode concluir-se que na Ria de Aveiro, particularmente na SML, existem comunidades bacterianas adaptadas à utilização se substratos hidrofóbicos, com uma boa representação de produtores de biossurfactantes. Os resultados confirmam a perspectiva de que a SML da Ria de Aveiro é um microhabitat com elevado potencial biotecnológico para isolamento de estirpes de bactérias hidrocarbonoclásticas produtoras de biossurfactantes com promissoras aplicações em processos de biorremediação de regiões estuarinas e costeiras após contaminação acidental com hidrocarbonetos de petróleo.

keywords

hydrocarbonoclastic bacteria, biosurfactant, bioremediation, petroleum hydrocarbons, ria de Aveiro

abstract

Bioremediation has proven to be an effective strategy in the recuperation of oil contaminated ecosystems. However most bacteria used in this processes, while being able to degrade a wide range of the oil hydrocarbons, have limited action due to the low water solubility of these compounds. Hence, a possible solution for this problem would be the use of biosurfactant-producing bacteria, since the presence of surfactants help improve the hydrocarbons dispersal, solubilization and bioavailability. The objective of this work was to assess the biotechnological potential of Ria de Aveiro estuarine system regarding the presence of hydrocarbonoclastic biosurfactant-producing bacteria and to evaluate different combinations of environmental inocula and carbon sources for the isolation of biosurfactants producing bacteria.

Selective cultures (diesel, crude and paraffin) were prepared using inocula from different environmental matrixes: samples from the surface microlayer (SML), bulk estuarine sediments and sediments of the rhizosphere of *Halimione portulacoides*, a characteristic halophyte from the salt marshes of Ria de Aveiro. During the incubation period, the development of the selective cultures was assessed by quantification of colony forming units (CFU). The highest value of CFU was obtained in the crude-sediment culture, while the lowest value was found with the diesel-rhizosphere combination. The DGGE profiles of the 16s rRNA gene fragments of the total community DNA extracted at the end of the incubation of the selective cultures, show that communities were different in terms of structural diversity. The values of the Shannon-Weaver index of diversity indicate that the higher diversity was achieved in the selective cultures with paraffin as carbon source (2.5231), followed by the crude oil (2.2509), and diesel (1.6726) selective cultures. From the selective cultures, 111 presumably hydrocarbonoclastic isolates were obtained after isolation and purification. Of these, 66 were tested for biosurfactant production by the atomized oil assay, with positive results for 17 isolates (25.8%). The environmental matrix with best results was the SML water and diesel was the most effective carbon source.

Having in consideration the high number of isolates obtained from the selective cultures and the percentage of biosurfactant producers, the estuarine system of Ria the Aveiro, and in particular the SML, can be regarded as an interesting seedbank for the prospection of hydrocarbonoclastic and biosurfactants producing bacteria. The SML microhabitat shows particularly high biotechnological potential for the isolation of bacterial strains with interesting properties for application in bioremediation strategies in coastal and estuarine areas.

Index

List of Images

List of Tables

Abbreviation Index

ASTM – American Society for Testing and Materials

BSA – bovine serum albumin

CFUs – colony forming units

CMC – critical micelle concentration

cSt – centistokes

DGGE – denaturing gradient gel electrophoresis

dH_2O – distilled water

DMSO – dimethyl sulfoxide

min. – minimum

max. – maximum

MSM – mineral salts medium

MW – molecular weight

OHCB – obligate hydrocarbonoclastic bacteria

PAHs – polycyclic aromatic hydrocarbons

PCR – polimerase chain reaction

PM – Pensky Marten

rDNA – ribosomal DNA

SDS – sodium lauryl sulfate

SML – sea surface microlayer

TSA – trypticase soy agar

TSB – trypticase soy broth

Tween 80 – polyoxyethylene (80) sorbitan monooleate

UV – ultra violet

1.INTRODUCTION

1 Introduction

1.1 Petroleum and its products

Petroleum is a form of *bitumen*[1] composed mainly of hydrocarbons and in nature is found in gaseous or liquid state, with *crude oil*, being the liquid part of petroleum [1].

The composition and characteristics of different types of crude oil vary considerably depending on the origin and age of the oil fields. These differences are due to the fact that although each kind of crude oil contains basically the same hydrocarbon compounds, the proportion of these hydrocarbons varies considerably from one crude to another [2].

Crude oil is a heterogeneous liquid, consisting of hydrocarbons comprised almost entirely of the elements hydrogen and carbon in the ratio of 1.85:1 respectively. It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3 % (v/v). There are also trace constituents, comprising less than 1 % (v/v), including phosphorous and heavy metals such as vanadium and nickel [1, 3]. The smallest hydrocarbon molecule in crude oil is methane (MW=16) and the largest are asphaltenes, with molecular weights in the tens of thousands. In between these two compounds there are thousands of others with a wide range of structural complexity: alkanes (or paraffins), cycloalkanes (or naphthenes or cycloparaffins), alkenes (or olefins), and arenes (or aromatics) [1].

The main characteristics of heavy hydrocarbons, such as those found in crude oils and heavy fuels, are variable density, low biodegradability, low solubility, low volatility, high viscosity and high sorption in soils. On the other hand, commercial hydrocarbons, which are lighter and are found in petrol and diesel fuel, have lower density than water, have higher biodegradability, generally have low solubility, although some fractions can be more soluble than others, highly or moderately volatile, low to medium viscosity and variable sorption in soils [4].

Diesel fuel is a product of petroleum distillation and contains on average 90 % aliphatic hydrocarbons and 10 % aromatic compounds. The most soluble fractions makes up to approximately 5 % by volume. It is possible to find very small traces of the following potentially dangerous compounds: benzene, toluene, xylenes and ethylbenzene [4]. More specifically, some

[1] "Native substances of variable color, hardness, and volatility, composed principally of the elements carbon and hydrogen and sometimes associated with mineral matter, the nonmineral constituents being largely soluble in carbon disulfide" [1].

batches of marine diesel oil (Table 1), a product of variable toxicity, can have a very high content of aromatic fractions (40 % or more). Other toxic materials such as phenols and sulphur compounds may also be present [5].

Table 1 – Specifications of Marine Diesel (Adapted from [2])

Property	Typical value
Flash point (PM closed)	338.7 K (min.)
Carbon residue	1.5 (max.)
Sulphur % wt	1.8 (max.)
Cetane number	30-58
Viscosity at 310.9 K	1.7-11.5 cSt
ASTM distillation – 90 % point	588.7-658.2K

1.2 Petroleum spillages and oil bioremediation approaches

Petroleum and its products are used at a global scale in almost all sort of modern activities. Such widespread use requires its constant transportation, which ultimately results in accidental or intentional leakages to the environment (Figure 1). The major causes for accidental oil spills are oil well blowouts, maritime accidents, pipeline ruptures, and explosions or leakages at storage facilities [6]. On the other hand, intentional releases such as operational discharges, that are often small, deliberate and "routine", can in most cases be controlled and/or avoided [7].

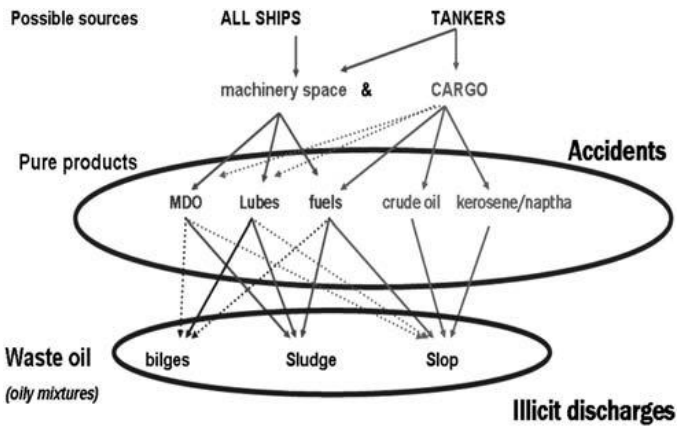

Figure 1 - Possible sources of oil spillage. MDO – Marine diesel oil [6].

The extent of the environmental and socio-economical impacts caused by an accidental oil spill can be determined by several factors [7]:

- The amount, rate and type of oil spilled;
- The location and the political and legal issues in place (which can influence the choice of the clean-up strategy adopted);
- The vicinity to sensitive resources;

- The choice and effectiveness of cleanup strategies.

Oil spills are one of major causes of ocean pollution caused by maritime transport of petroleum products, which most of the times end in ecological disasters of wide public concern [8, 9]. The resulting black tides can affect the environment in several ways. Although the groups that suffer the most are mainly birds, fish, sea mammals and several marine invertebrate species, due to negative effects in their physiology, immunology and development [10-12], some black tides can affect whole aquatic food chains [13]. As a consequence, oil spill can result in an accentuated decrease or disappearance of populations of marine fauna and flora within the affected area [14].

On land, the contamination of soils with petroleum or its products leads to a decrease on food productivity since it affects the germination and growth of some plants, as well as soil fertility [15, 16]. Also, crude oil contamination of land affects certain parameters of the soil, such as the mineral and organic matter content, the cation exchange capacity, the redox properties, and the pH [17].

Although large oil spills have higher ecological impacts than small scale events, it is also common for the later to have disastrous consequences in endangered species, especially when they occur in pristine areas [18]. It is also worth mentioning that, besides environmental damage, there are also the consequential high costs to fisheries, agriculture, related industries, and tourism in the affected areas [19].

Accidental spills have prompted the development of methodologies to deal with the resulting oil pollution both at sea and on land. These can be physical or chemical methods such as those illustrated in Figure 2, which are fairly well established, or biotechnological methods, which in the past decade have been subject to much debate and increasing research efforts. The later include such methods as the use of straw or plant material as an absorbent for oil, biosurfactants to clean oiled surfaces [20], biological polymers to coat surfaces and to prevent oil adhesion, and the addition of materials [21] (microorganisms or nutrients/fertilizers) to stimulate microbiological biodegradation of oil [22, 23].

This last procedure, also known as bioremediation, can be defined as the process of adding materials (nutrients - biostimulation or microorganisms - bioaugmentation) to contaminated environments under controlled conditions to cause an acceleration of the natural biodegradation processes until the contaminated site becomes innocuous or the contaminant reaches levels below the concentration limits established by the regulatory authorities [21, 24].

Successful bioremediation strategies can be a fairly complete solution to oil contamination, since the contaminants are converted into microbial biomass, water, and gases, primarily carbon dioxide, which form part of the carbon cycle [5, 25]. Physical methodologies only transfer the contaminant from one environmental compartment to another. For example, although some oil physically recovered from beaches may be recycled, in many cases it is stored in pits or landfills [22]. Additionally, in terms of the biota, bioremediation is one of the few processes that will actually remove toxic components from the environment. In fact, some field studies suggest that the natural rates of oil biodegradation on coastal shorelines can be stimulated two- to sevenfold by bioremediation strategies [22]. Nevertheless, it should be noted that bioremediation strategies are much slower to effectively remove the oil than intensive physical cleaning methods [22]. In cases of oil spills at sea, oil biodegradation rates are not sufficiently high, even when stimulated by the addition of nutrients or competent microorganisms, which makes this strategy unlikely to be used as a first response in these cases [26]. Bioremediation is also not the most adequate strategy to apply in cases where there are important environmental and/or political reasons to proceed with a quick decontamination. However, treatment options of the contaminated site should be considered regarding the overall benefits for the environment, weighting the gains of oil removal against the consequences of the cleanup strategy in each particular situation [22]. In this aspect, bioremediation tends to perform

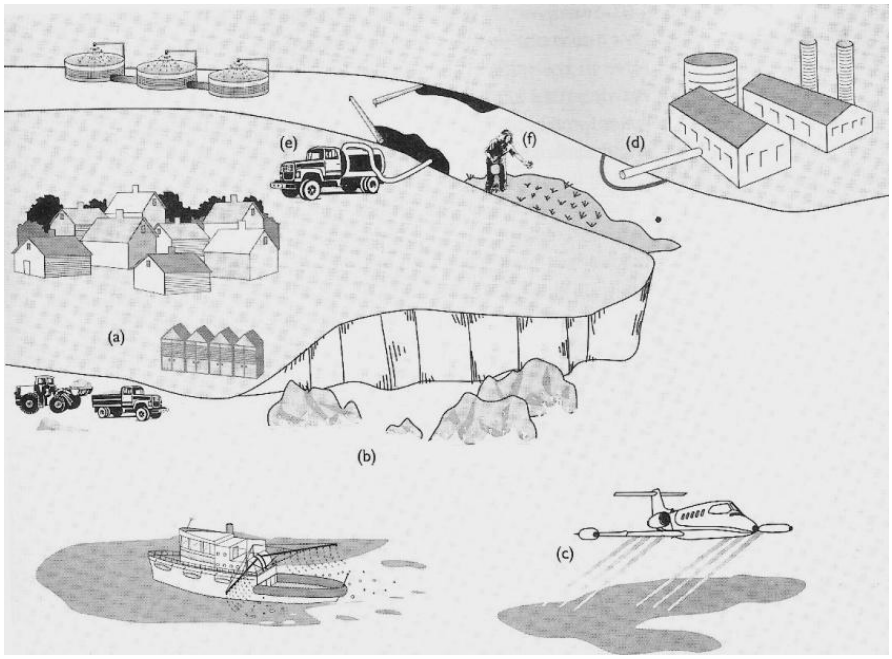

Figure 2 – Oil spill physical and chemical clean-up methods: a) beach clean-up by substratum removal; b) self-cleaning high energy shore; c) dispersant/surfactant spraying at the sea; d)booms protect cooling water intakes of industries and e) fish farms; f) hand cutting of oiled vegetation [127].

well, considering that its application can usually be conducted at low cost, with limited personnel, and with relatively little or no environmental impact [24, 27].

The success of bioremediation strategies depends on several factors. The most relevant are the type of contaminated matrix (soil/water), the penetration of the fertilizer and/or microorganisms in the impacted environment, the presence and concentration of biodegradable petroleum hydrocarbons in oil, the presence of other toxic compounds besides crude oil components, the level of available oxygen and nutrients, especially nitrogen and phosphorus, the biodiversity of hydrocarbonoclastic and cometabolising bacteria on site, the nature of the bioremediation product, and the prevailing environmental conditions, particularly temperature and pH [17, 22]. Oil-degrading microorganisms are ubiquitous in the environment [28, 29]. However, according to some studies, there is not a single microbial species that has the enzymatic ability to metabolize more than two or three classes of compounds typically found in a crude oil [30]. Therefore, consortia of several different bacterial species are often required to achieve significant levels of crude oil degradation after a spill [22]. Bioaugmentation strategies, involving a consortium of bacteria, are preferred to biostimulation in cases where pollutant toxicity or a lack of appropriate microorganisms, both in quantity and in quality, is important. The potential success of such strategy requires an understanding of the survival and activity of the added microorganism(s) and their genetics, as well as the knowledge of the general environmental conditions that control the biodegradation rates [23].

1.3 Oil-degrading bacteria

Hydrocarbon-degrading bacteria are widely distributed in marine habitats. Still, their roles in a natural marine environment or in oil-contaminated sites, with or without treatments such as the addition of fertilizers to enhance microbial activity, are largely unknown [31]. Some studies have shown that the introduction of oil or oil constituents into seawater leads to successive blooms of a relatively limited number of indigenous marine bacterial genera, usually obligate hydrocarbonoclastic bacteria (OHCB) (e.g. *Alcanivorax*), which are present at low or undetectable levels before the polluting event [32, 33]. As result, there is an increase of the degradation rate of many oil constituents, a process that can be accelerated further by the addition of the limiting nutrients [34]. Marine hydrocarbon degraders are usually highly specialized obligate hydrocarbon users, while terrestrial hydrocarbon degraders are more likely to be metabolically versatile and utilize a large range of organic substrates [31].

Some of the most common bacteria found in oil-impacted marine environments belong to the genus *Alcanivorax*, and have been proven to be important to the biodegradation of petroleum, especially under bioremediation conditions [35]. These Gram-negative marine bacteria are known to produce biosurfactants when using n-alkanes as substrate [36]. Additionally, they cannot use carbohydrates and amino acids as growth substrates [37]. *Alcanivorax borkumensis* is considered the paradigm of OHCB, and as such, its genome sequencing and functional genomic analysis has proven to be very helpful in understanding the genomic basis of the efficiency and versatility of its hydrocarbon utilization, the metabolic pathways used in hydrocarbon degradation, and its ecological success [31].

Other bacterial genera already known for their capacity to degrade hydrocarbons are *Pseudomonas, Marinobacter, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia* and *Gordonia [23]*.

The biodegradation potential of microorganisms is dependent of its genetic characteristics. Plasmids are thought to play a leading role in this aspect, since the ability to degrade more recalcitrant components of petroleum are generally plasmid generated [38]. Although catabolic plasmids are non-vital genetic elements, they provide a metabolic versatility not normally present in the cell (Table 2). Additionally, many of the bacterial catabolic pathways are associated to conjugative plasmids [39]. Since these plasmids are readily transferred laterally

Table 2 – Plasmids encoding catabolic functions [3].

Plasmid	Host	Compound(s) catabolised
TOL	*Pseudomonas putida*	Toluene, *p*- and *m*-xylene
NAH		Naphthalene
SAL		Salicylate
pND50		*p*-cresol
pWW31		Phenylacetate
pRE1		Isopropyl benzene
pAC25		3-chlorobenzoate
pAC21	*Pseudomonas* sp.	4- chlorobiphenol
pCIT1		Aniline
pWR1		3-chlorobenzoate
pCS1	*Pseudomonas diminuta*	Parathion
pJP1	*Alcaligenes paradoxa*	2,3- dichlorophenoxyacetic acid
pJP4	*Alcaligenes eutrophus*	2,4- dichlorophenoxyacetate and 3- chlorobenzoate
pKF1	*Acinetobacter* sp.	4- chlorobiphenol

into new host bacteria, other members of the community will acquire new degradation abilities leading to an increase of the metabolic potential in the ecosystem. It is such genetic potential that allows the evolution of integrated and regulated pathways for the degradation of hydrocarbons. Therefore, in an environment rich in a particular organic compound, the selective pressure may lead to the acquisition and maintenance of plasmids that encode the corresponding catabolic pathway [23]. For that reason, it is common to find degraders of exotic compounds in environments contaminated with such compounds. In some cases, the same catabolic genes may be located on a plasmid in one organism and on the chromosome in another, and these catabolic genes may influence the expression of other sets of catabolic genes present in the same cell [40].

1.3.1 Microbial degradation of oil hydrocarbons

Petroleum oil biodegradation by bacteria can occur under both aerobic and anaerobic conditions, although by the action of different consortia of organisms. In both cases an exogenous electron acceptor is needed for the degradation of hydrocarbons (being fully reduced substrates) by the microorganisms. In aerobic conditions, this electron acceptor is oxygen in the initial attack, and in most cases, also the electron acceptor of the subsequent steps [23]. In the subsurface, where there is low availability of molecular oxygen, oil biodegradation occurs primarily under anaerobic conditions. In these cases, biodegradation of partially oxygenated intermediates is mainly mediated by sulphate or nitrate reducing bacteria, or other anaerobes using other kinds of electron acceptors as the oxidant [23, 41]. Petroleum hydrocarbons are known to persist under strict anaerobic conditions, however in certain types of coastal marine sediments, under sulphate-reducing conditions some of these compounds are degraded. This kind of sediment when used to inoculate sediment that does not present the ability to degrade hydrocarbons under anaerobic conditions, induces the degradation of such components. This leads to believe that hydrocarbon contamination could be treated under sulphate-reducing conditions and that the inoculation with foreign microorganisms or equivalent samples of anaerobic microbial consortia adapted to specific hydrocarbons would be effective to stimulate the anaerobic biodegradation of these hydrocarbons [42, 43].

The inherent biodegradability of petroleum hydrocarbons depends on the chemical structures, physical state and toxicity of the compounds. For example, while *n*-alkanes are the group of petroleum components more readily biodegradable, the C_5-C_{10} homologues are toxic to the majority of hydrocarbonoclastic bacteria, since as solvents they tend to disrupt lipid membrane structures of microorganisms [44]. The fact that petroleum hydrocarbons, both in

crude oil and in refined products, occurs in complex mixtures, also affects the biodegradation rate of each component. This can have a positive or negative effect in the overall degradation process. In certain cases, some *iso*-alkanes are spared as long as *n*-alkanes are available as substrates, while, on other cases, some condensed aromatics are metabolized only in the presence of more easily utilisable petroleum hydrocarbons, a process referred to as co-metabolism [45].

As mentioned before there is a certain ubitiquity of gene clusters for the degradation of *n*-alkanes and (poly)aromatic fractions of oil in bacteria (Figure 3). For instance the *alk*SB1GHJ gene cluster, which encodes for enzymes involved in the degradation of *n*-alkanes, is present in *A. borkumensis* strain SK2 as well as in several other bacteria [31]. The *alk*SB1GHJ clusters of *Marinobacter aquaeolei* and *Oceanocaulis alexandrii* are flanked by putative transposase genes which is consistent with a previous observation that gene clusters for alkane degradation can be transferred among bacteria via mobile genetic elements [46].

As with alkanes, there is a similarity in the organization of genes associated to the degradation of aromatic hydrocarbons amongst different species of bacteria. Sequence analysis of *Cycloclasticus* sp. A5 revealed a cluster of *phn* genes involved in the degradation of naphthalene, methylnaphthalene, phenanthrene and dibenzothiophene. The *phnA1, phnA2, phnA3* and *phnA4* genes encode, PAH dioxygenase, the enzyme involved in initial step of the degradation pathway [47]. PAHs possessing four or more fused aromatic rings have very low water solubility and tend to be adsorbed to solid surfaces. These characteristics make them recalcitrant to biodegradation. However, some *Mycobacteria* and *Sphingomonas* are known to degrade these heavier PAHs [3, 35]. On the other hand, cycloalkanes, are degraded by a co-oxidation mechanism. These compounds are transformed by an oxygenase system to a corresponding cyclic alcohol, which is dehydrated into a cyclic ketone. Then, a monooxygenase system lactonises the ring, which is subsequently cleaved by lactone hydrolase. However, the two oxygenase systems usually never occur in the same organism, which makes necessary the use of synergistic actions by microbial communities to degrade cycloalkanes [48]. The degradation of substituted cycloalkanes seems to be less difficult than that of unsubstituted cycloalkanes [35].

1.3.2 Methods of isolation and characterization of oil-degrading bacteria

The initial method to isolate and identify petroleum-degrading microorganisms involved the use of hydrocarbons incorporated into agar-based medium [30]. The major problem of this approach is that in some studies, only a low percentage of the isolates from agar-based media could be demonstrated to actually be capable of hydrocarbon utilisation [23]. Therefore it is advisable to use media without agar, for example, silica-gel oil medium, as well as to have counts of petroleum degraders be expressed as a percentage of the total population rather than as total numbers of petroleum degraders *per se* [49].

An alternative to the plate technique for the estimation of hydrocarbon is the Most

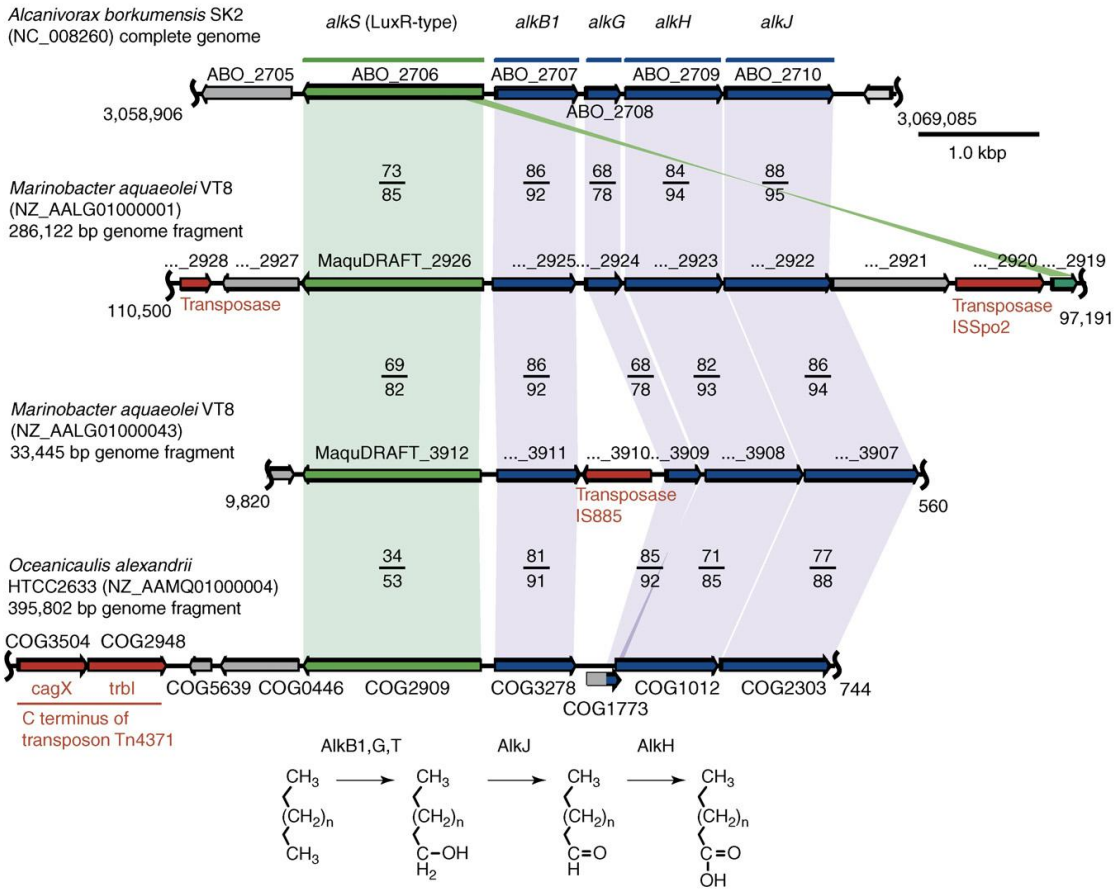

Figure 3 – Ubiquity of gene clusters among several bacterial species for the degradation of aliphatic fractions of oil. Organization of genes homologous to the *A. borkumensis alk* gene cluster in hydrocarbon-degrading marine proteobacteria. Homologous genes are highlighted by shaded areas: sequences predicted to code for LuxR-type transcriptional activators of the alkane genes, AlkS, are marked in green, genes for the alkane degradation pathway are indicated in blue, and transposase-related sequences are shown in red. Percentages of protein identity/similarity of polypeptides from *A. borkumensis* with those *of M. aquaeolei* and *O. alexandrii* are shown. Gene designations: *alk*B1, alkane monooxygenase; *alk*G, rubredoxin; *alk*J, alcohol dehydrogenase; *alk*H, aldehyde dehydrogenase [31].

Probable Number procedure, since it eliminates the need for a solidifying agent and allows the direct assessment of the ability to actually utilise hydrocarbons. It also has the advantage that the use of liquid media permits removal of trace organic contaminants and allows for the chemical definition of a medium with just a kind of hydrocarbon as carbon source. For these reasons, this technique allows to specifically count only hydrocarbon users as well as eliminate the problem of counting organism growing on other trace organic contaminants [50].

Although standard culture methods are adequate for the evaluation of the ability of indigenous bacteria to degrade contaminants, a significant problem of culture techniques is the fact that most (90-99 %) of the species making up competent degrading communities do not form colonies when laboratory-based culture techniques are used [51]. A way to avoid this problem is the use of molecular techniques for the analysis of the microbial communities that take part in *in situ* hydrocarbon biodegradation. The most commonly used are the measurement of lipid biomarkers, specifically, phospholipid fatty acids (PLFA), together with nucleic acid-based molecular techniques for fingerprinting the 16S rRNA component of microbial cells, such as DGGE [52, 53].

1.4 Surfactants

Surfactants are a diverse group of compounds that are economically important due to their tensioactive properties. They are mainly known for being the most common ingredient of detergent formulations and their primary function is to modify the interface between two or more phases in order to promote the dispersion of one phase into another. Surfactants are able to concentrate at interfaces due to their amphiphilic character, resulting from the combination of hydrophilic and hydrophobic moieties within the same molecule [54, 55]. Surfactants are generally characterized by properties such as the critical micelle concentration (CMC), chemical structure, charge, and in the case of biosurfactants, also by their source organism and the hydrophilic-lipophilic balance (HLB) [54].

The most common classification of chemical surfactants is according to the charge of their hydrophilic component: nonionic if the hydrophilic component is not ionized, anionic if the hydrophilic component comprises a negatively charged group, cationic if the hydrophilic component comprises a positively charged group, and amphoteric if the hydrophilic group contains both anionic and cationic characters [55].

A property of surfactants is their ability to form aggregate structures [54]. This characteristic is dependent on the concentration of the surface-active compounds until the CMC is obtained and will influence the surfactant activity [56]. Above the CMC the surfactant molecules aggregate to form micelles, bilayers and vesicles which lead to the reduction of the surface and interfacial tension and increase the solubility and bioavailability of hydrophobic organic compounds by partitioning the hydrophobic substrates and allowing closer cell-substrate interactions [57]. The aggregates may also fuse directly with microbial membranes resulting in direct substrate delivery [58]. However it is also possible that changes to the hydrophobicity of the cell surface my increase repulsion, [59], or the formation of micelles encapsulating the substrate may decrease bioavailability resulting in either reduction in toxicity or decreased bioavailability [60]. Therefore, the CMC of a surfactant is a commonly used characteristic to measure its efficiency [56]. Surfactants with lower CMC values are considered to be more efficient since less surfactant is needed to decrease the surface tension (Figure 4)[61].

Micelle formation also has a significant role in microemulsion formation [62]. This is particularly important in oil bioremediation strategies since microemulsions are clear and stable liquid mixtures of water and oil phases separated by monolayer or aggregates of surfactants, which are formed when one liquid phase is dispersed as droplets in another liquid phase, for example oil dispersed in water [61].

The HLB is another property of surfactants used to assess their effectiveness. This parameter is used to judge if a surfactant is related to oil-in-water or to water-in-oil emulsions, and specifies the portion of hydrophilic and hydrophobic constituents in surface-active substances

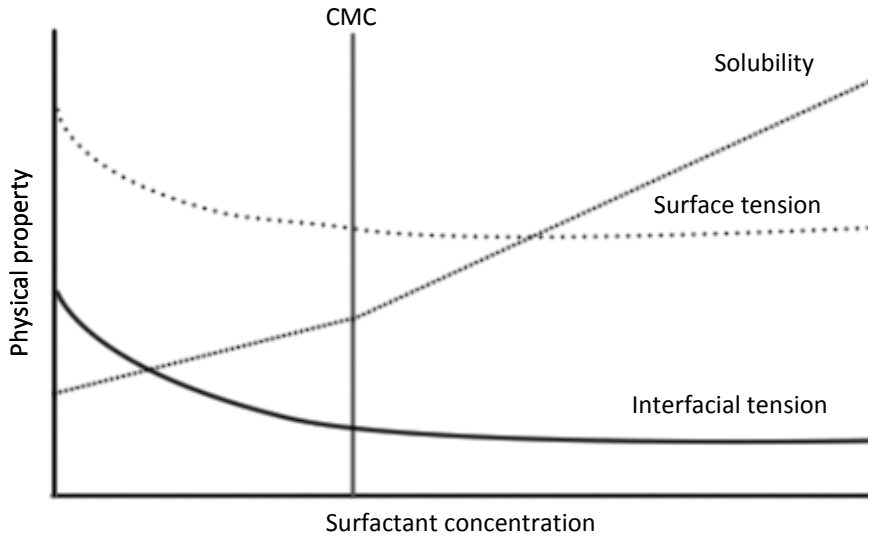

Figure 4 – Changes in surface tension, interfacial tension and solubility depending on surfactant concentration [68].

[56]. The HLB value also gives indication of the suitable applicability of a surfactant, making it useful information when planning bioremediation strategies. Surfactants with low HLB are lipophilic and stabilize water-in-oil emulsification, whereas surfactants with high HLB are hydrophilic and confer better water solubility [61, 63].

1.4.1 Bacterial biosurfactants

There are several microorganisms that are able to synthesise a wide range of surface-active compounds, generally called biosurfactants. Therefore, biosurfactants are molecules that possess the same characteristics of chemical surfactants but are produced by microorganisms. These compounds are mainly classified according to their molecular weight, physico-chemical properties and mode of action [56]. The low-molecular-weight biosurfactants (e.g. lipopeptides, glycolipids and proteins) reduce the surface tension at the air-water interfaces and the interfacial tension at oil-water interfaces, whereas the high-molecular-weight biosurfactants, also called bioemulsifiers (e.g. polymers of polysaccharides, lipopolysaccharides proteins and lipoproteins) are more effective in establishing oil-in-water emulsions [64].

The best studied biosurfactants are the glycolipids, which include the rhamnolipids (Figure 5), the trehalolipids, sophorolipids and mannosylerythriol lipids. These compounds contain mono- or disaccharides, combined with long-chain aliphatic acids or hydroxyaliphatic acids. It is also important to refer that the major producers of rhamnolipids belong to the *Pseudomonas* genus [64].

In microorganisms, biosurfactants are naturally involved in microbial competitive interaction, cell-to-cell communications, plant and animal pathogenesis, increasing of bioavailability of surface-bound subtracts and heavy metals (via direct interfacial contact and pseudosolubilization), avoidance of toxic elements and compounds, motility and biofilm formation and maintenance [54, 64]. Usually they are secondary metabolites, produced at the end of the log phase and/or stationary phase.

When compared with synthetic surfactants, biosurfactants have several advantages, such as, higher rates of biodegradability, lower toxicity, better foaming properties and better stability at extreme pH,

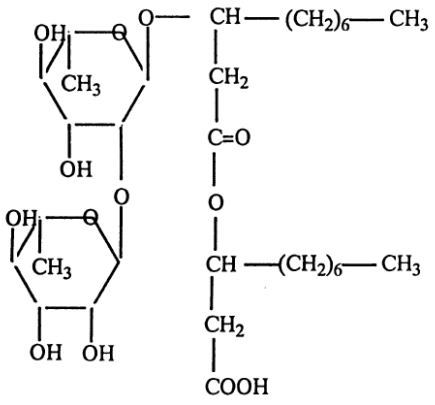

Figure 5 – Chemical structure of a rhamnolipids [132].

salinity and temperature values [65-67]. However, despite all these environmentally favourable characteristics, their commercialisation has not yet been fully achieved, since its production costs cannot compete with those of the chemical surfactants [64].

1.4.2 Biosurfactants and oil bioremediation

A possible application of biosurfactants is in the remediation of environmental contamination with organic compounds, such as hydrophobic hydrocarbons. In this case, due to the fact that the contaminant is hydrophobic, there is relatively little area of contact between the water phase and the oil phase. In these circumstances the addition of biosurfactants aims to increase the bioavailability of the hydrocarbons (biosurfactant-enhanced bioremediation) or the mobilisation and removal the contaminants by pseudosolubilization and emulsification in a washing treatment [35, 64]. Biosurfactant addition to contaminated sites may also increase substrate bioavailability by increasing the hydrophobicity of the surface of the cell allowing hydrophobic substrates to associate more easily with bacterial cells [68]. As mentioned before, the production of biosurfactants can be activated as a response of the microorganisms to the need to use water insoluble substrates. In these cases biosurfactant production is necessary for specific adhesion mechanisms to large oil drops or for the emulsification of oil, followed by the uptake of submicron oil droplets [69, 70]. These mechanisms are present in many oil-utilising microorganisms, which produce cell wall associated or extracellular surface-active agents, to facilitate the hydrocarbon uptake through the hydrophilic outer membrane [71]. Such agents are mainly low molecular weight compounds, such as fatty acids, triacylglycerols and phospholipids.[3, 72] Cultures of these bacteria become brown and turbid as the oil slick is transformed into many small oil droplets. Bacterial cells are associated on the surface of the droplets, and such contact may facilitate the assimilation of petroleum components into the cells [35].

Several studies and field trials have proved that the introduction of external surfactants in contaminated environments will influence the degradation rate of oil components [3]. However, while in some cases the effect is stimulatory [73-76], in others it has proven to be inhibitory or neutral [77, 78]. This, added to the fact that chemical dispersants have caused further ecological damage to the ecosystems after application for removal of spilled oil, makes the use of biosurfactants, which are less toxic and partially biodegradable, preferable to use in bioremediation strategies [23, 79].

1.5 Environmental matrixes as a seedbank for surfactant-producing hydrocarbonoclastic bacteria

1.5.1 The sea surface microlayer

The sea surface microlayer (SML) represents the interface between the ocean and the atmosphere, where the transfer of material is controlled by complex physicochemical processes. It is considered as the uppermost 1st mm of the water column, and presents distinct physicochemical properties compared with subsurface water. This interface can serve as a sink of anthropogenic compounds, such as petroleum hydrocarbons, due to its unique chemical composition, enriched in biogenic organic molecules, such as lipids, proteins and polysaccharides [80]. Because of the particular features of the SML, such as the presence of the surface film and the high surface tension, it is considered to be a unique habitat and the living communities that develop in this compartment of the water column are referred to as the neuston. Bacterial communities that are present in the surface microlayer are known as the bacterioneuston. These microorganisms are subjected to both favorable (e.g. high concentrations of organic and inorganic nutrients) and detrimental factors (e.g. intense UV radiation, high concentrations of heavy metals and organic pollutants, temperature and salinity fluctuations). As a result, the SML is considered to be an extreme environment for microorganisms, which explains the occurrence of unusual species and taxa. Some of the bacterial strains isolated from SML are Proteobacteria and Actinobacteria, and include genera such as *Pseudomonas, Chromobacterium, Aeromonas* and *Micrococcus* [81, 82].

In areas associated with anthropogenic coastal activities, particularly shipping harbors, it is common to find high levels of PAHs in SML water. Results of several studies lead to believe that the amount of PAHs in the SML in these sites is related to the size of the port and intensity of shipping traffic [83]. The main causes are related to the discharge of waste water form shipping (proximity of the contamination source) combined with limited water exchange in the harbor (restricted hydrodynamics) [84-86]. However, atmospheric deposition of combustion residues and biogenic sources may also lead to the enrichment of PAHs in the SML [83].

1.5.2 Rhizosphere environments

Plant roots are known to have important effects on the structural diversity of bacterial communities inhabiting soil and sediment closely attached the roots (rhizosphere effect).[87]. The microorganisms present in rhizosphere communities may have negative, positive or neutral

effects on the plant, influencing its growth and health [88]. The accumulation of microorganisms in the rhizosphere is supported by root exudates which make the surrounding soil rich in sugars, amino acids, hormones, sugar alcohols, organic acids, vitamins, terpenoids, coumarins and flavonoids [89]. However these secretions may also lead to a decrease of certain microbes [87]. On the other hand the microbial community present in the rhizosphere deeply influences the plant by decomposition, affecting nutrient uptake, antagonistic effects in other microorganism and parasitism [88].

Microorganisms in the rhizosphere are mostly found in biofilms rather than in the planktonic state [90]. However, bacterial interactions, growth and formation of biofilms on the root and sediment surfaces have been shown to involve complex mechanisms [91] and in some cases it is connected to biosurfactant production [92, 93]. In the rhizosphere, biosurfactant production has also been associated with swarming motility, zoosporicidal and antifungical activities [93-95] .

1.5.3 Bulk sediments environments

Soil not directly influenced by root activity is referred as bulk sediment or soil [87]. Most of the microbial communities present in bulk sediments are also found in rhizosphere. However, since in most cases there are less nutrients available in bulk soil than in the rhizosphere, commonly bulk sediment communities have lower diversity and quantity of microorganisms [87, 96]. Estuarine sediments in particular, are known sinks of contaminants [97], with petroleum hydrocarbons being the most common contaminants [98].

Microorganisms in sediments are fundamental to diagenesis of organic matter. An important ability involved in such processes is their capacity to form biofilms, on thesurface of sediment particle [99]. As in rhizosphere, the formation of biofilms by bulk sediment bacteria is associated with the production of biosurfactants [100, 101].

1.6 Goals and strategy

Bioremediation has proven to be an environmental friendly and economically feasible strategy for recuperation of oil contaminated ecosystems. However the application of this technique is often limited by the hydrophobicity of the contaminants, making them hard to be metabolized by the bacteria used. Hence, a possible solution for this problem would be the use of hydrocarbonoclastic biosurfactant-producing bacteria, since the presence of surfactants help improve the hydrocarbons dispersal, solubilization and bioavailability.

Thus, the main goal of this study was to assess the biotechnological potential of hydrocarbonoclastic biosurfactant-producing bacteria isolated from the estuarine system of Ria de Aveiro (Portugal). Selective cultures of hydrophobic hydrocarbons were prepared to stimulate the growth of potential hydrocarbonoclastic biosurfactant-producing isolates, using three different carbon sources (Arabian light crude oil, maritime diesel and liquid paraffin) and three different environmental matrixes (SML water, bulk sediment and rhizosphere) from Ria de Aveiro. The communities established in the different selective cultures were characterized in terms of the concentration of CFU and compared in terms of structural diversity by analysis of 16S rRNA DGGE profiles. A set of isolates was retrieved, typed by BOX-PCR and tested for biosurfactants production by the atomized oil assay.

2. MATERIAL AND METHODS

2. Material and Methods

2.1 Characterization of the study area and sampling site

Ria de Aveiro is a shallow estuary-coastal lagoon system, located in north Portugal at approximately 40.7° N, 8.7° W. The physical system is characterized by many branching channels that are connected to the Atlantic Ocean by a single tidal channel [102].

All samples were collected on the 25th October 2010 at a sampling site (40°37'32.0952" N, 8°44'9.2832" W) located at the east margin of Mira channel, one of the main branches of the estuarine system of Ria de Aveiro (Figure 6). The Barra salt marsh encompasses approximately 2.2 hectares of vegetated area [103] and is located in the proximity of the commercial port of Aveiro, and several smaller harbors used by leisure and fishing ships. Analysis of sediment samples (superficial horizon 0-5 cm) of this site have shown that it is mainly constituted by clay and has a mean value of organic matter of 9 % [104]. The most common salt-marsh vegetation species found at this site are *Halimione portulacoides* (L.) Aellen, *Sarcocornia perennis* (Miller) A.J. Scott subsp. *perennis*, *Salicornia ramosissima* J. Woods, *Juncus maritimus* Lam. and *Limonium vulgare* Miller [105]. The rhizosphere of *H. portulacoides* harbors a dense bacterial community, because this particular micro-habitat is thought to favorably meet bacterial requirements in terms of the availability of labile organic compounds [106]. Colonized sediments of the Ria de Aveiro are richer in fine particles and in inorganic nutrients, namely nitrogen and

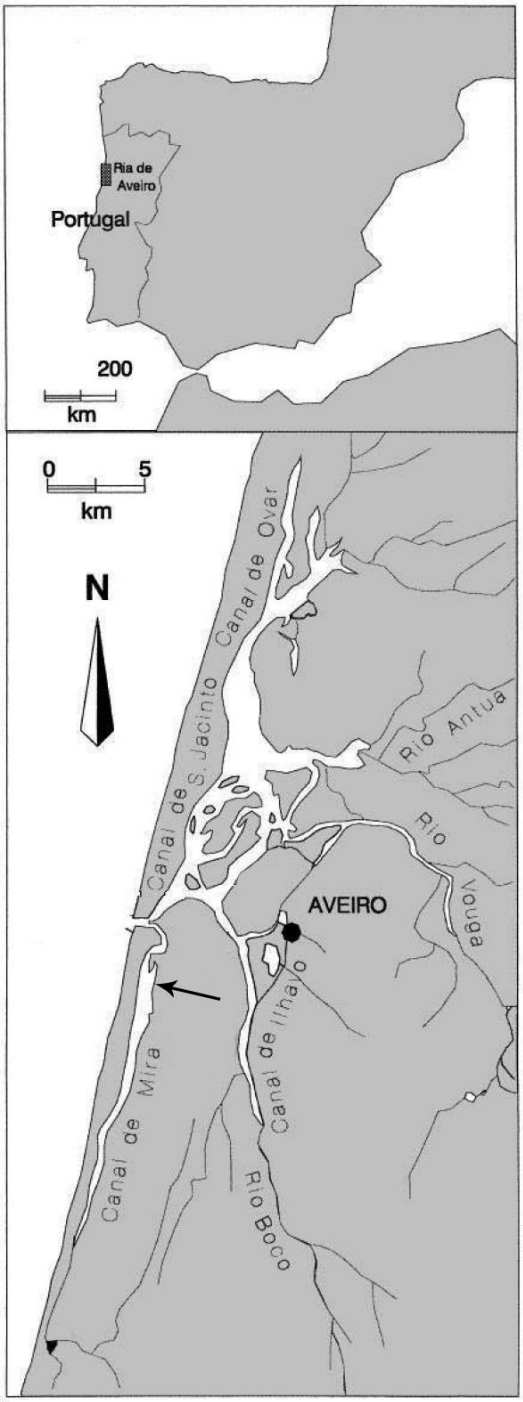

Figure 6 – Ria de Aveiro (Portugal). Arrow indicates sampling site.

phosphorus, than the nonvegetated area [102], which can provide for the main limiting nutrients for hydrocarbonoclastic bacteria.

2.2 Sampling

2.2.1 Sea surface microlayer (SML)

The SML sample was collected according to an adaptation of the glass plate method [107]. For that, a plexiglas plate was disinfected with ethanol (70 %), rinsed with sterilized dH_2O and finally repeatedly immerse in water from the sampling site, immediately before the sampling. For collection of the SML, the plate was slowly submerged in an upright position, slowly removed in the same position (Figure 7-A). After allowing the plate to drip for 5 seconds, the water adhering to both surfaces was removed by forcing the plate between two Teflon wiper blades and collecting the water in sterilized glass bottles (Figure 7-B). This process was repeated several times until approximately 50 mL of SML water was collected.

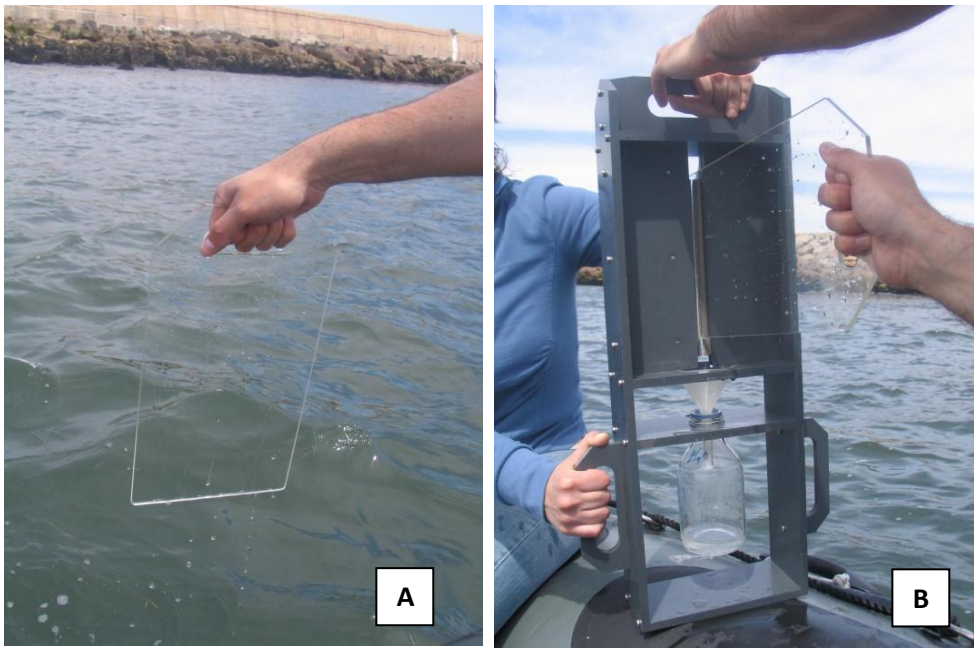

Figure 7 – SML sampling procedure. A – Slow immersion and submersion of the Plexiglas plate in the water; B- collection of the SML water in a sterilized glass bottle.

2.2.2 Sediment

The sediment sample was obtained by scraping the upper 3 cm of unvegetated sediments at the lower limit of the intertidal area at the margin of Mira Channel with a sterilized spatula. The

sediments were transferred to sterilized plastic bags and brought to the laboratory in isothermal boxes.

A previous study has determined that the sediment used present a water content of 17.3 % in relation to sediment fresh weight and has classified the sediment as very fine silt sand with the fraction of fine particles corresponding to 35.2 % of the sediment dry weight [108].

2.2.3 Rhizosphere

Rhizosphere samples were obtained from a monospecific stand of the halophyte plant *Halimione portulacoides.* Intact plant specimens were collected and the excess sediment was gently removed in the field. The plants were transferred to sterilized plastic bags and brought to the laboratory in isothermal boxes.

Oliveira *et al.* [108] determined that the sediment of *Halimione portulacoides* banks present at 3-4 cm a water content of 22.2 % for the month of September. In the same study the sediment was classified as mud with the fraction of fine particles corresponding to 94.8 % of the sediment dry weight.

2.3 Extraction of microbial cells from the environmental matrixes

In the laboratory, the roots of *Halimione portulacoides* specimens were carefully washed with distilled water in order to remove the loosely attached sediment. After washing, the roots were cut in 2-4 cm fragments with the aid of sterilized scissors and forceps.

For the extraction of microbial cells, sub-samples of 5 g of sediment or 5 g of root were placed in Erlenmeyers with 45 mL of extraction solution (0,1 % Tween 80, Merck; and 0,1 % sodium pyrophosphate) and 5 g of glass beads (4 mm diameter). The suspensions were placed on a shaker incubator (model SI4-2, Shel Lab, U.S.A.) for 5 min at 100 rpm. After the agitation, particles were allowed to settle for 1 min. and the supernatant was used as inoculum [109].

The water of the SML did not suffer any process for extraction of microbial cells and was directly used as inoculum.

2.4 Culture media

Mineral Salts Medium (MSM), was obtained from a Base Mineral Medium (BMM, [110]) consisting of 1.0 g L^{-1} NH_4NO_3, 0.7 g L^{-1} KCl, 0.0005 g L^{-1} $FeCl_3 \cdot 6H_2O$, $2x10^{-5}$ g L^{-1} $CaCl_2$, $5x10^{-6}$ g L^{-1} $CuSO_4$, $5x10^{-6}$ g L^{-1} $MnCl_2 \cdot 4H_2O$ and $1x10^{-4}$ g L^{-1} $ZnSO_4 \cdot 7H_2O$ (Sigma), prepared with brackish water

(17 g/L NaCl, AppliChem; pH 7.2 ± 0.2) to which 2 % agarose (Gentaur) was added and solubilized by heating to 100 °C for 5 min. The culture medium was then sterilized in the autoclave (20 min, 121 °C). After sterilization, the medium was allowed to cool down to approximately 45-50 °C and the thermo labile compounds were added as sterilized stock solutions in order to obtain de final concentrations of 3.0 g L^{-1} Na_2HPO_4 (Sigma), 7.0 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 2.0 g L^{-1} KH_2PO_4 (Sigma); pH 7.2 ± 0.2, and 0.1 g L^{-1} cicloheximide (VWR). All reagents were purchase from Merck except when otherwise indicated. For liquid cultures, MSM was prepared without agarose.

2.5 Carbon sources

For this work, three hydrocarbon-rich carbon sources were tested: liquid paraffin (Merck), light Arabian crude oil and maritime diesel. Before being added to the mineral medium, the carbon sources were sterilized. by the following methods: paraffin was autoclaved for 20 min at 121 °C; a small volume of Arabian light crude oil was transferred to a petri dish in order to obtain a thin layer that was exposed to UV radiation for 4 hours; maritime diesel was sterilized by vacuum filtration at 300 mmHg using PTFE membranes with Ø 0.2 µm pore size.

2.6 Selective cultures with hydrocarbons

To select for biosurfactant producing hydrocarbonoclastic bacteria, hydrophobic carbon sources were used in the selective cultures using the different environmental inocula (Table 3). The cultures were prepared in sterile erlenmeyers by adding 1 mL (1 %) of the sterilized carbon source and 5 mL (5 %) inoculum (SML water or sediment/rhizosphere cell suspensions) to 94 mL of liquid MSM. The nine cultures were incubated in an orbital incubator at 26 °C and 130 rpm for 2 months. At the end of the incubation, sub-samples of each culture were transferred to 50 mL Falcon tubes and preserved at -16 °C for DNA extraction and metagenomic analysis.

Table 3 – Designation of the several selective cultures in relation to the inoculums environmental matrixes and the hydrophobic carbon sources

		Hydrophobic carbon sources		
Inoculum		Arabian light crude oil	Maritime diesel	Liquid paraffin
	SML water	CA	DA	PA
	Bulk sediment	CS	DS	PS
	Rhizosphere sediment	CR	DR	PR

2.7 Colony forming units (CFUs)

Starting from the third week, the concentration of viable bacteria was assessed by the number of CFUs in non selective medium. Weekly, 100 µL of each culture were serially diluted in Ringer solution and aliquots of 100 µL of the appropriate dilution range were spread plated in triplicate in Tryptic Soy Agar (TSA) plates (Merck; 15 g/L casein peptone, 5 g/L soy peptone, 5 g/L NaCl, 15 g/L agar; pH 7.3±0.2) containing 0.1 g/L cicloheximide (VWR) to restrain the growth of fungi. The cultures were incubated at 28 °C for 2 days, after which the colonies were counted and the concentration of CFUs was estimated from the average counts in the most suitable dilution and corrected for the dilution factor.

Statistical tests were performed using SPSS v15.0. Differences between the CFUs mL^{-1} results obtained between the various carbon sources and matrixes through time were analyzed by two-way repeated measures ANOVA. Sphericity of the data was evaluated by the Mauchly's sphericity test. If the data met this criteria ($p>0.05$) it was directly subjected to parametric analysis of variances with Bonferroni adjustments, if not ($p<0.05$) Greenhouse-Geisser correction was used.

2.8 Isolation and purification of bacterial strains

Every week, during the 2 months incubation period of the selective cultures, 3 replicates of 100 µL of each culture were serially diluted in Ringer solution and spread-plated on solid MSM on which 50 µL of the corresponding carbon source had been previously spread. The plates were incubated at 25 °C for 2 weeks. From the plates of the most convenient dilutions of each combination of carbon source and environmental matrix, isolated colonies were selected based on morphology and color. For further isolation and purification, the selected colonies were streak plated in solid MSM amended with the corresponding carbon source. The plates were then incubated at room temperature (approx. 25 °C) for a week and the purification by streak platting was repeated 3 times. Isolates were then inoculated with a sterile loop in liquid MSM medium with 1 % of the respective carbon source. When the purity of the isolates was confirmed by optical microscopy after gram staining, the cultures were frozen with glycerol (15 %; AppliChem) and kept at 4 °C until processing.

2.9 Denaturating gradient gel electrophoresis (DGGE)

2.9.1 DNA extraction of selective cultures

Total DNA was extracted from the 2-month selective cultures according to the protocol described by Costa *et al.* [111]. A total volume of 10 mL of each of the selective was centrifuged at 13,300 rpm for 5 min. The pellet was ressuspended in 500 µL of ethanol 96 %, mixed in the vortex and then transferred to Fast Prep tubes containing a mixture of beads with different diameters. The bead mixture was composed by 0.1 g of each kind of the following beads: 0.75-1.0 mm, 0.1 mm, 0.25-0.5 mm and 2.85-3.45 mm glassbeads; and 0.1 mm zirconia/silica beads. Previous to their use the glass beads were washed in the Fast Prep tubes. This consisted in covering the beads in the tubes with HCl solution (5.8 mM), mixing it well and incubating at room temperature for 1 hour, with occasional agitation. The HCl solution was then removed with a micropipette and sterile dH_2O was added to cover the beads. The beads were washed by agitating the tube for 30 s and dH_2O was removed. The washing procedure was repeated 4 times. Finally the tubes were slightly opened and placed in an incubator at 65 °C overnight to dry. For the extraction, the tubes containing the bead mixture and the samples were agitated for 2 consecutive periods of 30 seconds in the FastPrep FP120 bead beating system (Qbiogene, USA) at 5.5 m/s.

The suspensions were then transferred to new microtubes and centrifuged at 13,300 rpm for 5 min. The supernatants were discarded and 1.2 mL of extraction buffer (100 mM sodium phosphate [pH 7.0], 100 mM Tris-HCl [pH 7.0], 100 mM EDTA [pH 8.0], 1.5 M NaCl, 1 % CTAB and 2 % SDS [112]) was added to the pellet. The mixture was gently homogenized then incubated at 65 °C for 30 min. After a new centrifugation at 13,300 rpm for 5 min, the supernatants were transferred to new tubes containing 1 mL of 21:1 chloroform-isoamyl alcohol solution. The tubes were carefully mixed and then centrifuged at 13,300 rpm for 5 min. The resulting aqueous phase was then transferred to a new microtube containing 0.6 % isopropanol (vol/vol) and incubated for 30 min at room temperature. After a final centrifugation at 13,300 rpm for 5 min, the resulting pellet of nucleic acids was washed twice with 0.5 mL 70 % cold ethanol and then air dried. The pellet was resuspended in 40 µL of TE buffer (10 M Tris-HCl, pH 7.4 containing 1mM EDTA.Na_2, pH 8) and stored at - 20 °C. All centrifugations were performed on a Heraeus Pico 17 centrifuge (Thermo Scientific).

2.9.2 PCR-amplification of 16S rRNA gene fragments

For PCR-amplification of the 16S rRNA gene fragments of the community DNA extracted from the selective cultures the *primers* U27F (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´) [113] synthesized by IBA (IBA GmbH, Germany) were used. The composition of the reaction mixture (25 µL) was 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix (Fermentas), 0.25 µL of each primer, 1.25 µL BSA (2 g L^{-1}; Sigma) and 9.75 µL dH_2O. The PCR cycle was composed by 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94 °C, 45 s at 56 °C, and 1.5 min at 72 °C, and a final extension step at 72 °C for 10 min.

The PCR products were then submitted to *nested* PCR. The *Bacteria* domain specific primers used in the second PCR are 968F -GC (5´-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3´) and 1401R (5´-CGG TGT GTA CAA GAC CC-3´) [114] synthesized by IBA (IBA GmbH, Germany). The reaction mixtures had a total of 25 µL and were made of 0.5 µL sample, 12.5 µL DreamTaq™ PCR Master Mix (Fermentas), 0.5 µL of each primer, 2.0 µL acetamide (Fluka) and 9.0 µL dH_2O. For the second PCR reaction, the denaturation step took 4 min at 94 °C, followed by 30 thermal cycles of 1 min at 95 °C, 1 min at 53 °C, and 2 min at 72 °C, and finally an extension step at 72 °C for 7 min. PCR reactions were conducted in a MultiGene Gradient Thermal Cycler (MIDSCI).

The success of the amplification of the 16S rRNA gene fragments was verified by agarose (Gentaur) gel (1.8 %) electrophoresis, with $5.3x10^{-6}$ % (vol/vol) GelRed (Biotium) as DNA staining agent, at 80 V for 30 min in 1x TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA; Fluka; pH 8.0). The presence of bands was visualized in a UV transiluminator (Benchtop UV).

2.9.3 DGGE run and analysis

DGGE was performed with the DCode System (C.B.S. Scientific). PCR products containing approximately equal amounts of DNA were loaded onto 6-9 % (w/v) polyacrylamide gel in 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA; Fluka; pH 8.0). The 6-9 % polyacrilamide gel (bisacrylamide:acrylamide = 37.5:1) was made with a denaturing gradient ranging from 32 to 60 %. Electrophoresis was performed for 16 h at 150 V at 60 °C in 1 x TAE buffer. Following electrophoresis, the gels were silver-stained [115]. Briefly, gels were fixed with 0.1 % (v/v) ethanol plus 0.005 % acetic acid (Merck), stained with 0.3 g $AgNO_3$ (Merck) and developed with 0.003 % (v/v) formaldehyde (Merck) and 0.33 % Na OH (9 %)(Merck). A 0.75 % Na_2CO_3 (Fluka) solution was used to stop the development.

The bands with higher intensity or that were present in several lanes of different samples were cut from the gel and stored in microtubes at -20 °C for later cloning and identification.

The profiles obtained were analyzed with GelCompar 4.0 software (Applied Maths, Belgium). The bands occupying the same position in the different lanes of the gels were identified. A binary (1/0) matrix was constructed taking into account the presence or absence of individual bands in each lane. Cluster analysis was performed using PRIMER v5 software (Primer-e, UK). The binary matrix was transformed into a similarity matrix (dendrogram) using the Bray Curtis measure. In order to compare the diversity between the different selective cultures, the Shannon–Weaver diversity index (H) was calculated as follows: $H=-\Sigma(pi)\ (\log 2pi)$, where i represents all the unique bands and pi is the relative abundance of band i [116] using software PRIMER v5.

2.10 Molecular typing of the isolates by BOX-PCR

2.10.1 DNA extraction of bacterial isolates

Aliquots of glycerol-amended frozen cultures were used to inoculate 1 mL of TSB (Merck) in a microtube, which was incubated overnight at 26 °C in an orbital shaker (model SI4-2, Shel Lab), at 120 rpm. The procedure for DNA extraction was based in the protocol described by Henriques *et al.* [117]. 500 µL of culture was transferred to new microtubes and centrifuged at 13,300 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 100 µL of TE buffer (10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA) and 50 µL of 30 g L^{-1} lysozyme solution (Roche). After incubation at 37 °C for 40 min, 50 µL of lyse solution (Genomic DNA Purification Kit, Fermentas) was added. The mixture was incubated at 65 °C for 10 min and after this period 20 µL of SDS 25 % was added. The mixture was further incubated at 65 °C for 10 min more after which 50 µL of 5 M NaCl was added. The mixture was agitated in the vortex at maximum speed for one minute and 200 µL of chloroform was added. The mixture was homogenized by repeated inversion of the tubes and then centrifuged at 13,300 rpm for 5 min. The aqueous phase was then carefully pipetted to new microtubes in which 100 µL of isopropyl alcohol was added and then mixed by inversion. Finally the mixture was centrifuged at 13,300 rpm for 5 min and 100 µL of cold ethanol 96 % was added and mixed by inversion. After an incubation of 15 min at 4 °C the mixture was again centrifuged at 13,300 rpm for 5 min. The supernatant was removed and the DNA-RNA pellet was washed with 100 µL of ethanol 70 %. The reaction mixture was allowed to settle for 1 min and again centrifuged at 13,300 rpm for 3 min. The supernatant was removed and

the microtubes were open so that the pellet could dry at room temperature. Finally the DNA-RNA pellet was resuspended in 50 µL of TE buffer and the microtubes were kept at -20 °C. All centrifugations were performed on a Heraeus Pico 17 centrifuge (Thermo Scientific).

2.10.2 BOX-PCR procedures and analysis

BOX-PCR was conducted in order to identify isolates with similar genotypes and to group them in clones according to their similarity in order to spot suitable representatives for identification, thus reducing the DNA sequencing effort. The PCR mix used was composed of 13.25 µL dH_2O, 2.5 µL KCl buffer 10x, 2.5 µL dUTPs, 3.75 µL $MgCl_2$, 1,25 µL DMSO, 0.25 µL Taq polymerase, 0.5 µL *primer* BOX A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and 1.00 µL template for 25.00 µL of reaction (all reagents from Fermentas). The cycling conditions used included a denaturation step of 7 min at 94 °C, followed by 35 thermal cycles of 1 min at 94 °C, 2 min at 53 °C, and 8 min at 65 °C, and finally an extension step at 65 °C for 16 min. PCR was conducted in a MyCycler Thermal Cycler (BioRad). The PCR products were stored at -20 °C.

The PCR products were then run in agarose (Gentaur) gel (1.5 %) electrophoresis, with $5.3x10^{-6}$ % (vol/vol) GelRed (Biotium), at 80 V for 3h in TAE buffer 1x (5Prime). The profiles were visualized in a UV transiluminator (Benchtop UV) and were photographed using a Canon Powershot G10. The profiles obtained were analyzed with GelCompar 4.0 software (Applied Maths, Belgium). A dendrogram relating all isolates was generated by clustering correlation using the UPGMA method with fine alignment.

2.11 Analysis of biosurfactant production

The isolates were tested as to their capacity to produce biosurfactants by the *atomized oil assay* [118]. This technique has proven to be more sensitive to lower amounts of biosurfactants than the commonly used *drop-collapse* method [118], and has also the advantage of allowing a quick screening of a high number of isolates.

The isolates used were initially in liquid selective cultures (MSM with 1 % of the respective carbon source). Not all isolates grown in liquid selective cultures were considered to be pure by gram staining, and therefore due to time limiting factors and so that a more comprehensive analysis of the production of biosurfactants was conducted, the isolates that were grown in non-selective TSB for the BOX-PCR analysis were also tested. As such some isolates were tested twice, from the rich medium and from the selective culture, so that a preliminary study of the preference for rich or selective media for the production of biosurfactants by the bacteria could

be conducted. However for the calculus of the biosurfactant producers percentage all replicas of an isolate were counted as one. With the aid of a sterilized toothpick, the isolates were spotted on plates of Luria Bertani (LB) agar (10 g/L tryptone, Merck, 5 g/L yeast extract, Merck, 10 g/L NaCl, AppliChem, and 15 g/L agar, Liofilchem; pH 7.0 ± 0.2) and incubated overnight at 28 °C. *Escherichia coli* DH5α was used as a negative control [118] and solutions of commercial surfactants were used as positive controls: 0.008 mM Tween 80 (Merck), 10 mM SDS (BioRad), and 1.19 g L^{-1} surfactin (Sigma). Aliquots of 2 µL of each surfactant solution were spotted on the surface of the LB agar plates. The analysis was performed by using an airbrush (model BD-128P, Fengda, China) to nebulize liquid paraffin (Merck) at a constant range over the inoculated LB agar plates. The presence of biosurfactants was detected by the immediate appearance of a halo surrounding the colony, visualized under indirect bright light.

3. RESULTS

3. Results

3.1 Selective cultures with hydrophobic substrates

Nine different selective cultures were prepared with varying carbon sources and inocula. The original appearance of the liquid cultures was of an aqueous phase in the bottom and a very thin organic phase consisting of the hydrophobic substrate added as carbon source at the surface. During the course of the incubation, the organic phase became less distinct and the aqueous phase became turbid, especially in the crude and paraffin cultures (Figure 8).

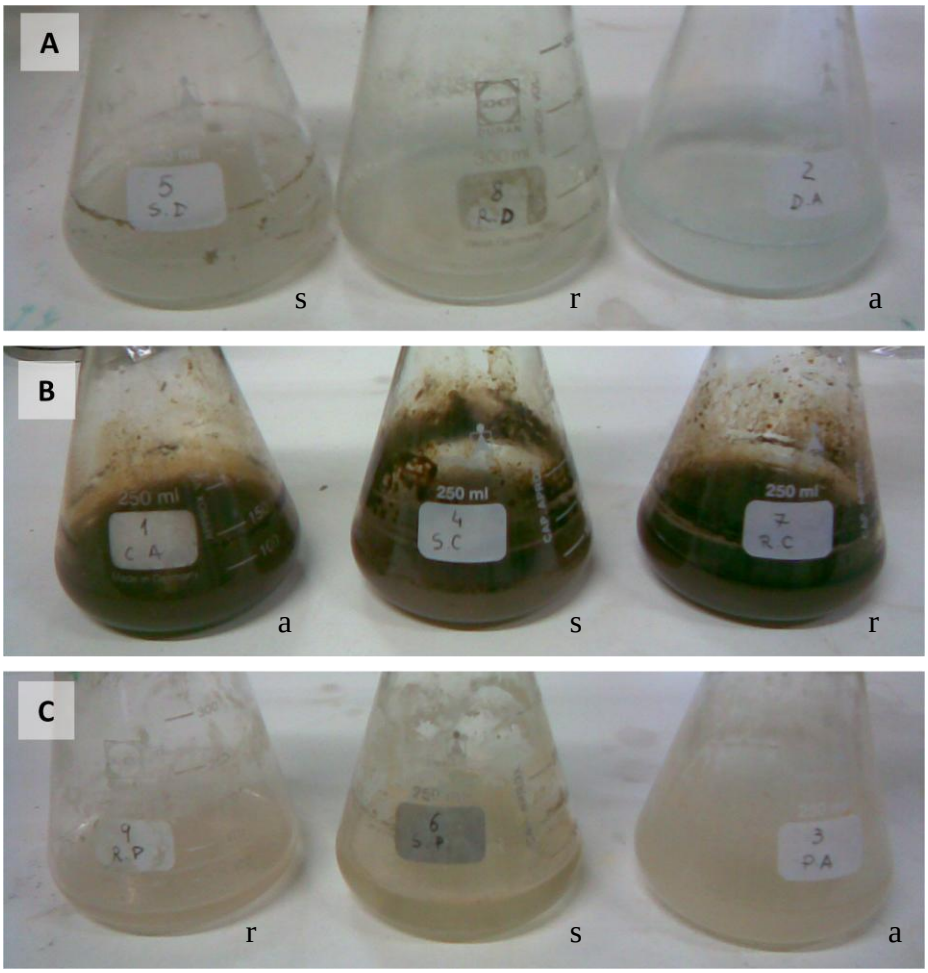

Figure 8 – Selective cultures using hydrocarbon–rich substrates as hydrophobic carbon sources, (A) maritime diesel, (B) Arabian light crude oil, and (C) liquid paraffin, were used for isolation of biosurfactants producing bacteria inhabiting SML water (a), bulk sediment (s) and rhizosphere (r), after 2 months of incubation at 130 rpm at 26°C.

3.2 Bacterial abundance of selective cultures

The variation of the concentration of CFU/mL in the selective cultures from week 3 until the end of the incubation are represented in Figure 9, Figure 10 and Figure 11.

The selective culture with highest overall concentration of CFUs throughout the incubation period was the CS culture (average of $2{,}77 \times 10^{8}$ CFU mL^{-1}), and the lowest was found in the DR culture (average of $1{,}73 \times 10^{7}$ CFU mL^{-1}). Through statistical tests (two-way repeated measures ANOVA) all crude, diesel and paraffin cultures with different matrixes were shown to be significantly different between themselves ($p<0.05$) except between CA and CR and PS and PR ($p>0.05$). In the crude cultures (Figure 9) the number of CFUs showed a tendency to decline over time, while in the diesel cultures (Figure 10) a relatively constant level of CFUs was maintained in the last 4 weeks, and in the paraffin selective cultures (Figure 11), with the exception of PS, a slight tendency of the CFUs values to increase was observed in the last weeks.

All rhizosphere cultures (Figures 9, 10 and 11) have the lowest CFUs values, although in a relatively steady level throughout the incubation period. Statistical tests have shown that there is no significative difference between CR and PR regarding bacterial abundance ($p >0.05$). Bulk sediment and SML water selective cultures present similar levels of CFUs except for the cultures with crude as carbon source in which the sediment culture had significantly higher CFU concentration. Statistical tests have found all sediment cultures to be significativily different among themselves regarding bacterial abundance ($p<0.05$), as well as the SML water cultures except between PA and DA ($p >0.05$). Bulk sediment selective cultures and SML water showed high CFU concentration in the first weeks and a steep decrease, followed by a relative stabilization in the later weeks of the incubation period, except for the PS and CA cultures. Post hoc tests confirmed this results showing that at the third week bulk sediment cultures presented significativily higher bacterial abundance than at the fifth week ($p<0.05$). The SML water cultures also presents significativily higher CFU values for the fifth week in comparison to the last three weeks ($p<0.05$).

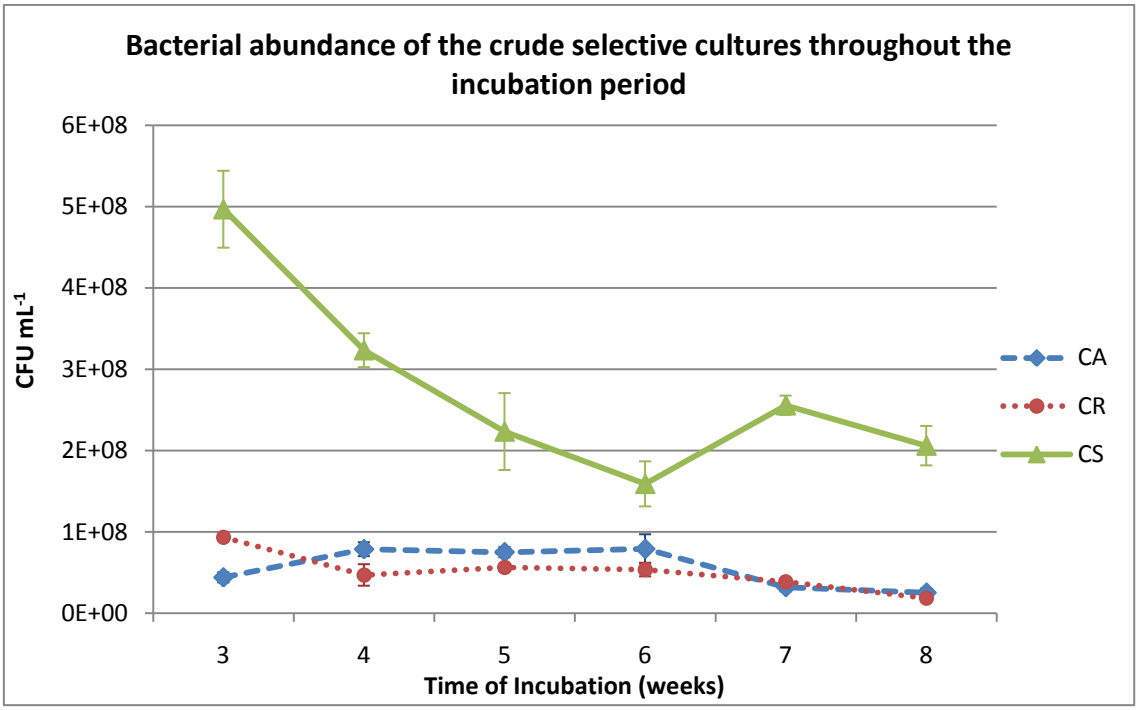

Figure 9 – Variation of the concentration of CFU between the third week and the end of the incubation in selective cultures containing light Arabian crude oil as carbon source and SML water (CA), rhizosphere sediment (CR) and bulk sediment (CS) as inocula. The values correspond to the average of the 3 replicates of the most suitable dilution and the error bars represent the standard deviation.

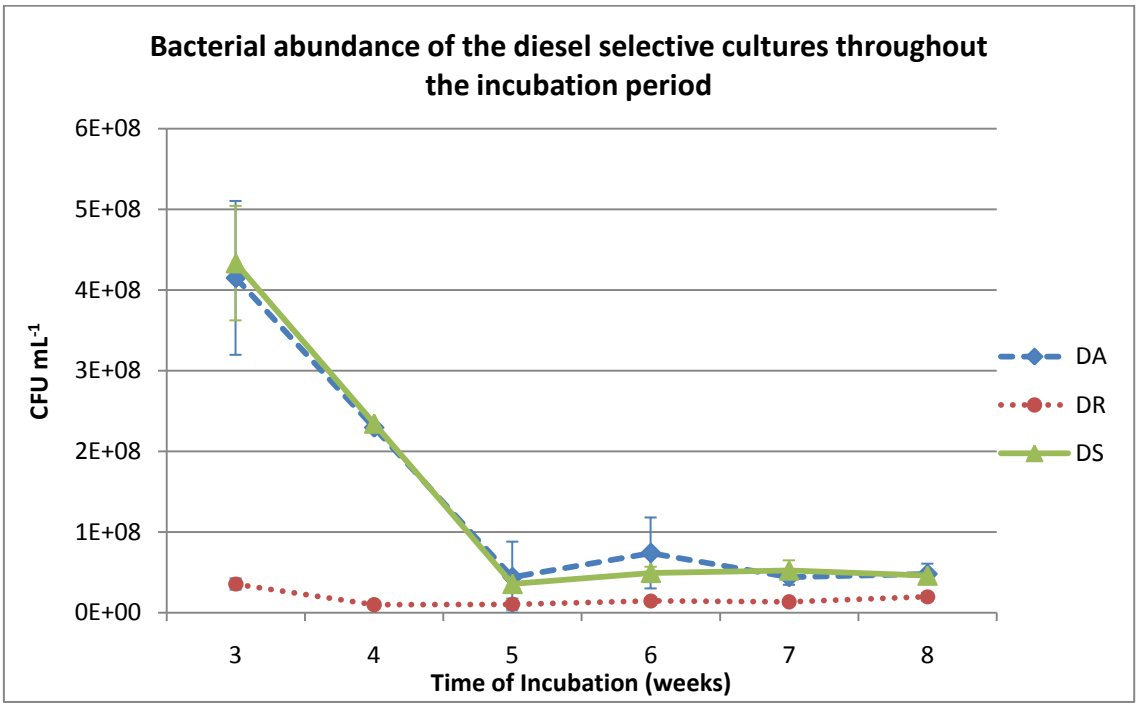

Figure 10 – Variation of the concentration of CFU between the third week and the end of the incubation in selective cultures containing maritime diesel as carbon source and SML water (DA), rhizosphere sediment (DR) and bulk sediment (DS) as inocula. The values correspond to the average of the 3 replicates of the most suitable dilution and the error bars represent the standard deviation.

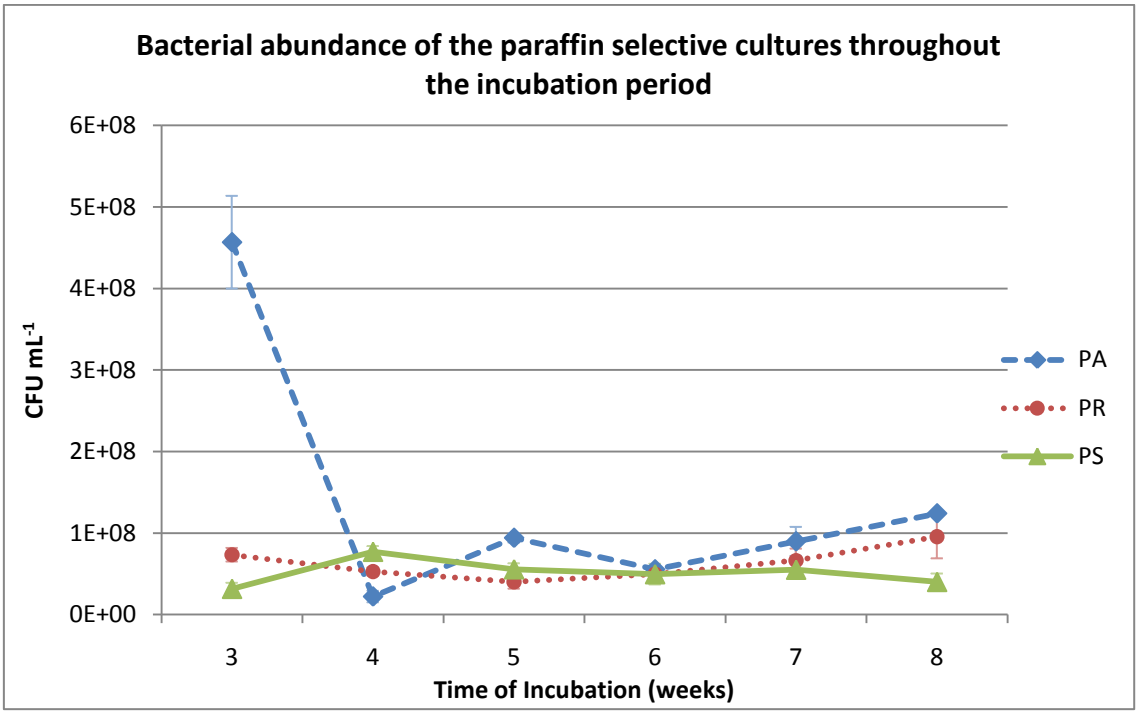

Figure 11 – Variation of the concentration of CFU between the third week of and the end the incubation in selective cultures containing liquid paraffin as carbon source and SML water (PA), rhizosphere sediment (PR) and bulk sediment (PS) as inocula. The values correspond to the average of the 3 replicates of the most suitable dilution and the error bars represent the standard deviation.

3.3 Isolation and purification of colonies

Having in consideration the different carbon source used, morphology and color of the colonies in selective medium, an initial set of 111 isolates was collected during the 2 months of incubation of the 9 selective cultures. Later, the 111 isolates were inoculated in liquid selective media only 32 could be maintained and kept pure in selective media. From the remaining that were found to be contaminated or extremely slow-growing in selective media, an additional set of 34 isolates could be retrieved from the non-selective TSB cultures used for the BOX-PCR analysis. Finally a set of 66 isolates was achieved. During the isolation and purification procedures some isolates obtained from the crude selective culture produced colonies surrounded by a clearance zone when grown on crude-amended solid medium (Figure 12).

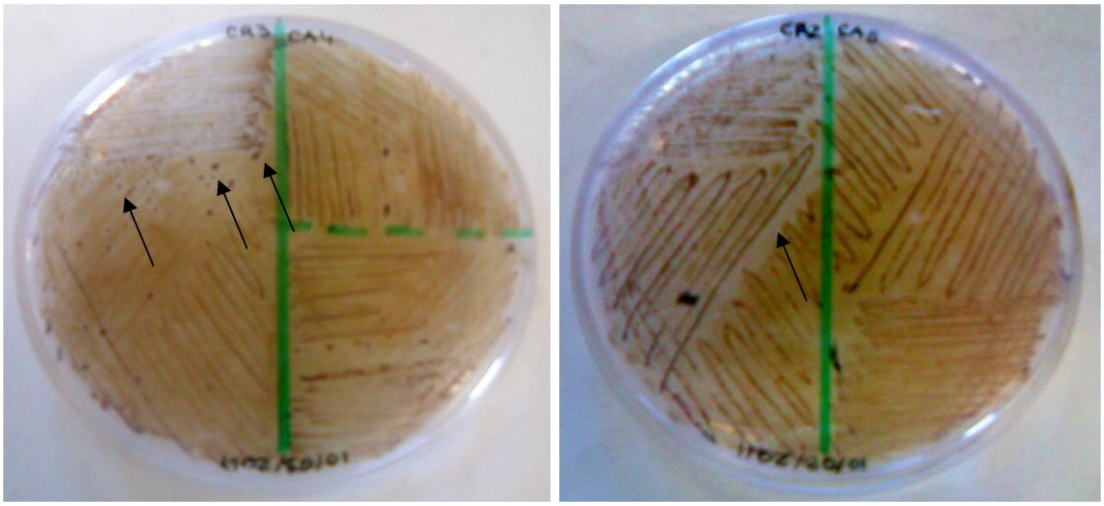

Figure 12 – Examples of streak-plating cultures of bacterial isolates obtained from the crude selective medium, in crude-amended MSM agar. Colonies have a brownish color contrasting with the white color of the medium and the clear zones surrounding some isolated colonies are indicated with arrows.

3.4 Denaturing gradient gel electrophoresis (DGGE)

The DGGE profiles obtained from the separation of products of amplification of 16s rRNA gene fragments of the different environmental matrices used as inocula are displayed in Figure 13.

The dendrogram generated with the software PRIMER v5 from the DGGE profiles is presented in Figure 14.

Two crude cultures (CA and CR) show a degree of similarity of approximately 40% and present little similarity with other samples. There are also two paraffin cultures (PR and PA) with a degree of similarity of approximately 60 %. In other cultures, there was not enhanced similarity in cultures with the same carbon source. Two sediment cultures (DS and PS) presented a similarity of a little over 60 % although amended with different carbon sources. The SML cultures were little similar among them, while all the sediment cultures share a minimum of 50 % similarity.

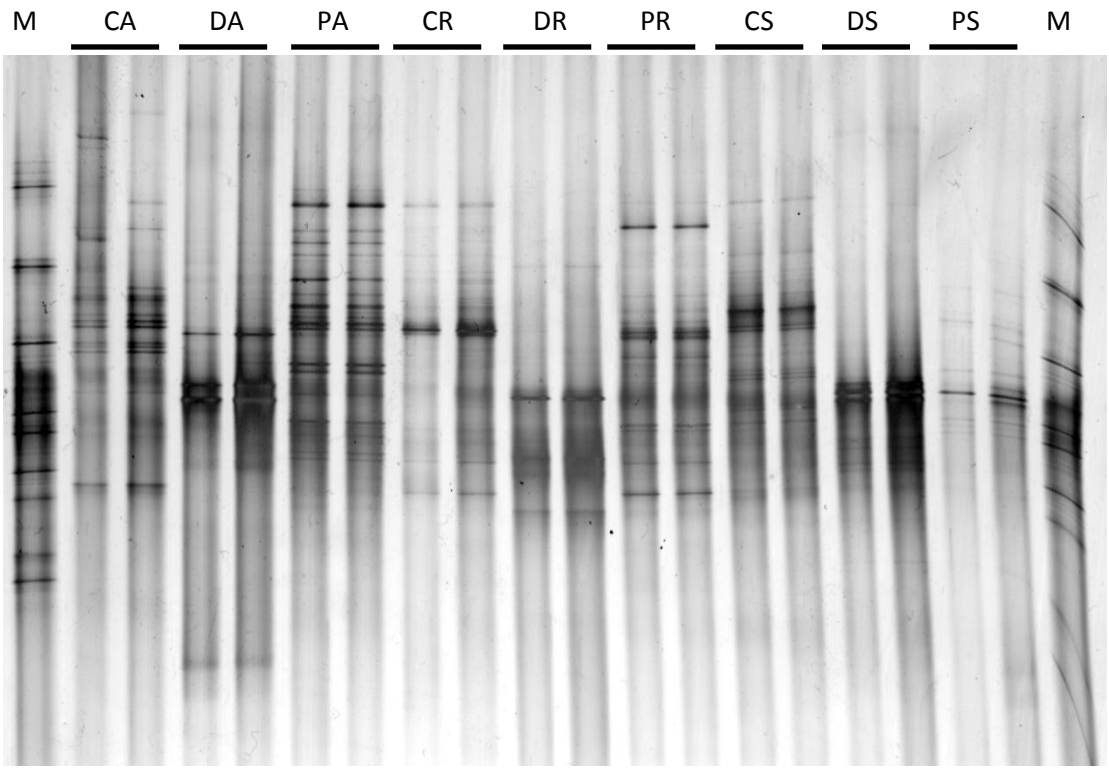

Figure 13 – DGGE profiles of 16s rRNA genes fragments amplified from the selective cultures of SML water (A), rhizosphere sediment (R) and bulk sediment (S) matrixes samples from the estuarine system Ria de Aveiro after 8 weeks of incubation with crude (C), maritime diesel (D) or liquid paraffin (P) as hydrophobic carbon sources. M – marker.

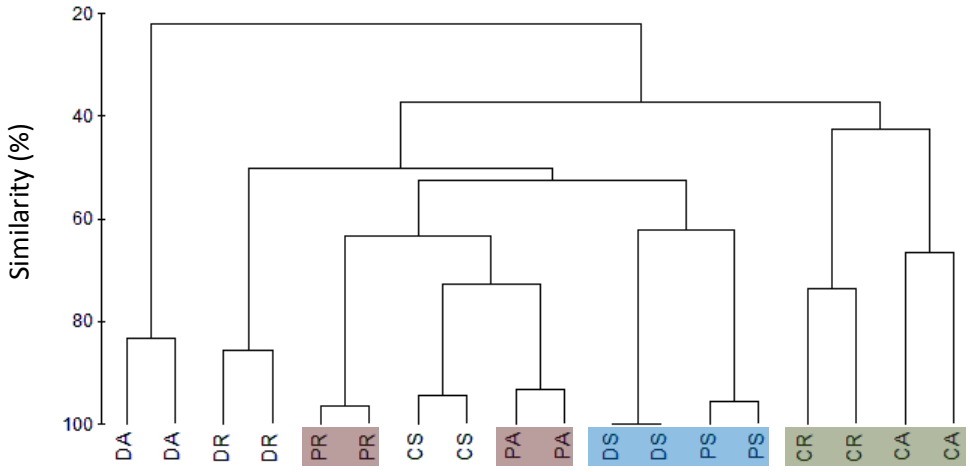

Figure 14 – Dendrogram representing the different selective cultures according to the structural similarity inferred by DGGE analysis of 16SrDNA sequences. Pairs of cultures with relevant percentages of similarity are highlighted. Carbon sources: (D) Maritime diesel, (C) Arabian light crude oil, (P) Liquid paraffin; Matrixes: (A) SML water, (S) bulk sediment and (R) rhizosphere.

With the same data (number and position of bands representing different ribotypes) the Shannon-Weaver index of diversity was calculated for each selective culture (Table 4). The highest diversity was found in the selective culture of SML water containing paraffin as carbon source (PA). The average Shannon-Weaver index average is 2.5231 for the paraffin cultures, 2.2509 for crude cultures and 1.6727 for diesel cultures. Averaging by inoculum type, the Shannon-Weaver index of diversity is 2.3581 for bulk sediment, 2.2877 for SML water, and 1.8248 for rhizosphere.

Table 4 – Shannon-Weaver index values of the selective cultures from the samples sorted from the more diverse to the less diverse communities as inferred from the analysis of the DGGE profiles with PRIMER v5. Carbon sources used in the cultures: (D) Maritime diesel, (C) Arabian light crude oil, (P) Liquid paraffin; Matrixes used to inoculate the cultures: (A) SML water, (S) bulk sediment and (R) rhizosphere.

Selective cultures	PA	CS	PR	CA	DS	PS	CR	DA	DR
Shannon-Weaver Index	2,9134	2,6006	2,4933	2,4468	2,2392	2,1626	1,7053	1,5029	1,2759

3.5 Genetic typing of the isolates by BOX-PCR

Genetic typing of the subset of 66 isolates resulted in the formation of 59 different genotypes (Figure 15). The isolate 36 was used a positive control through different electrophoresis gels. Since the similarity of the controls (isolate 36) is 90 % at the minimum, a cutoff of approximately 90 % was assumed as the threshold for identical strains. Five clusters of isolates with more than 90 % similarity (approx.) were identified.

3.6 Screening of the isolates for biosurfactant production

From the 111 isolates acquired through isolation and purification in MSM solid medium only the remaining 32 pure isolates in liquid selective medium and the 66 isolates in TSB used in BOX, were tested for the production of biosurfactants. Of these two groups there is an intersection of 32 isolates and in total 66 different isolates were tested.

Of the 66 overall isolates tested with the *atomized oil assay* 17 were positive (25.8 %) for the production of biosurfactants. Since the aim of this test is to identify biosurfactant producers, the isolates that produced biosurfactants in one medium but not in the other were counted in the overall percentage as biosurfactant producers, that is, as a positive result. Those that presented just negative or positive results in both media were counted just once. From the total of 66 different isolates tested, 4 positive results came from both media, 5 from the TSA medium and 11 from the MSM subset.

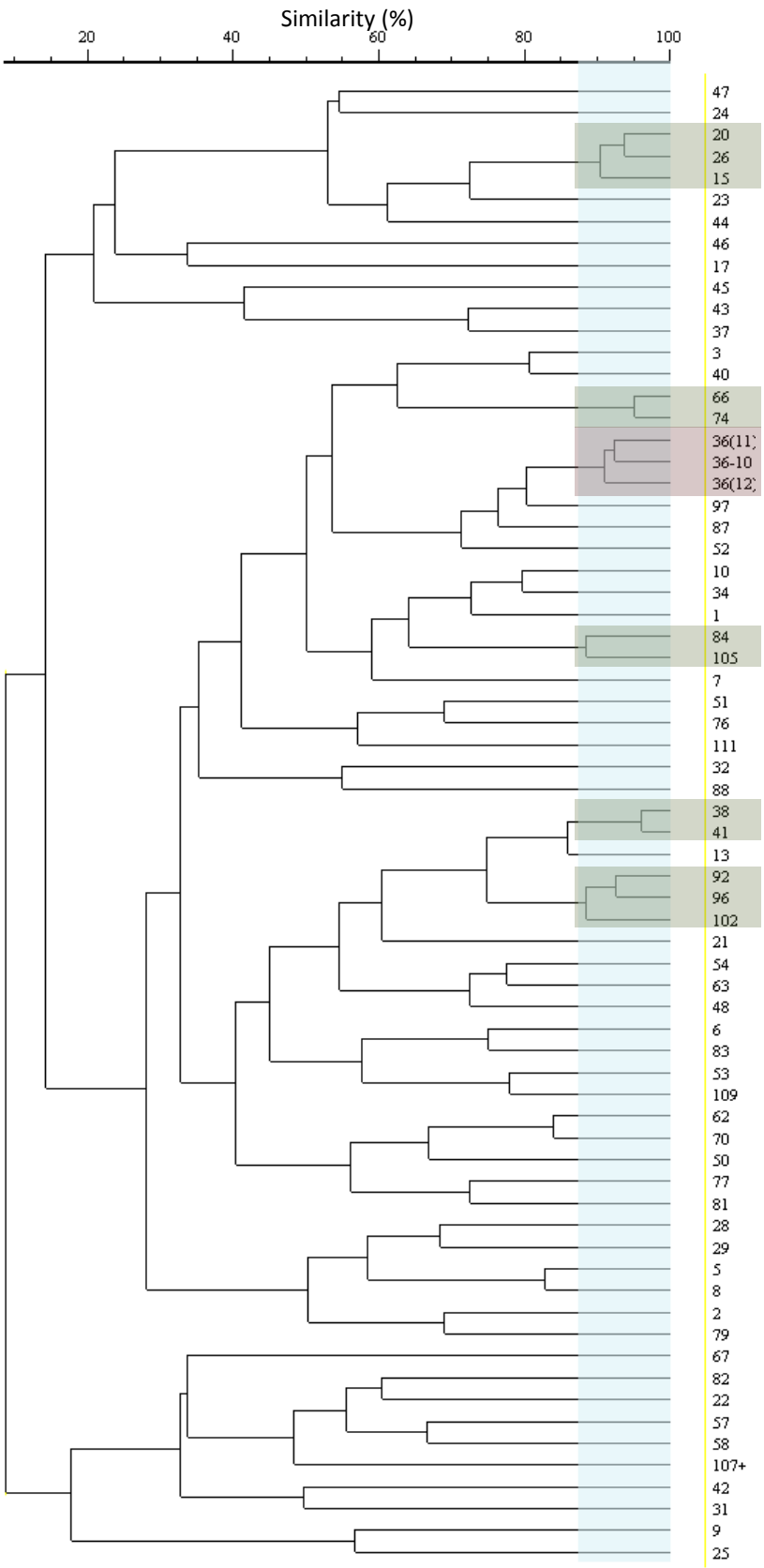

Figure 15 – Dendrogram of the results from the electrophoresis of the BOX-PCR products. Each number represents an isolate. Blue area represents the threshold for identical strains. Isolates with a similarity percentage within the shadowed area are considered identical, green highlight. Isolates used as controls are highlighted in pink.

In the subset of 32 isolates tested from both media 5 isolates from the TSA medium had positive results (15.6 %) while from the MSM isolates 11 were positive (34.4 %). 4 isolates were positive independently of the medium of origin.

The percentages of positive results by carbon sources and ambient matrixes are presented in Table 5 . Considering the total percentages, the highest frequency of biosurfactants producing strains was observed in SML isolates and the lowest in isolates from the sediment cultures. Comparing the different carbon sources, biosurfactants production was more frequent in the subset of isolates obtained from the diesel cultures and less frequent among strains isolated from paraffin amended cultures.

Table 5 – Percentage of biosurfactant-producing isolates in relation to the original inoculum and to the hydrophobic carbon sources. A total of 66 different isolates was tested with the *atomized oil assay.* Between brackets is the absolute number of isolates with positive results.

	SML	Sediment	Rhizosphere	Total
Crude	25.0 %(1)	0.0 %(0)	44.4 %(4)	25.0 %(5)
Diesel	66.7 %(4)	11.1 %(1)	28.6 %(2)	31.8 %(7)
Paraffin	20.0 %(1)	20.0 %(2)	22.2 %(2)	20.8 %(5)
Total	40.0 %(6)	11.5 %(3)	32.0 %(8)	

Examples of biosurfactants producing colonies and of the negative and positive controls are displayed in Figure 14. Positive results correspond to the immediate development of a halo around the biosurfactant producing colonies or around the area where a drop of solution of commercial surfactants/biosurfactants was spotted.

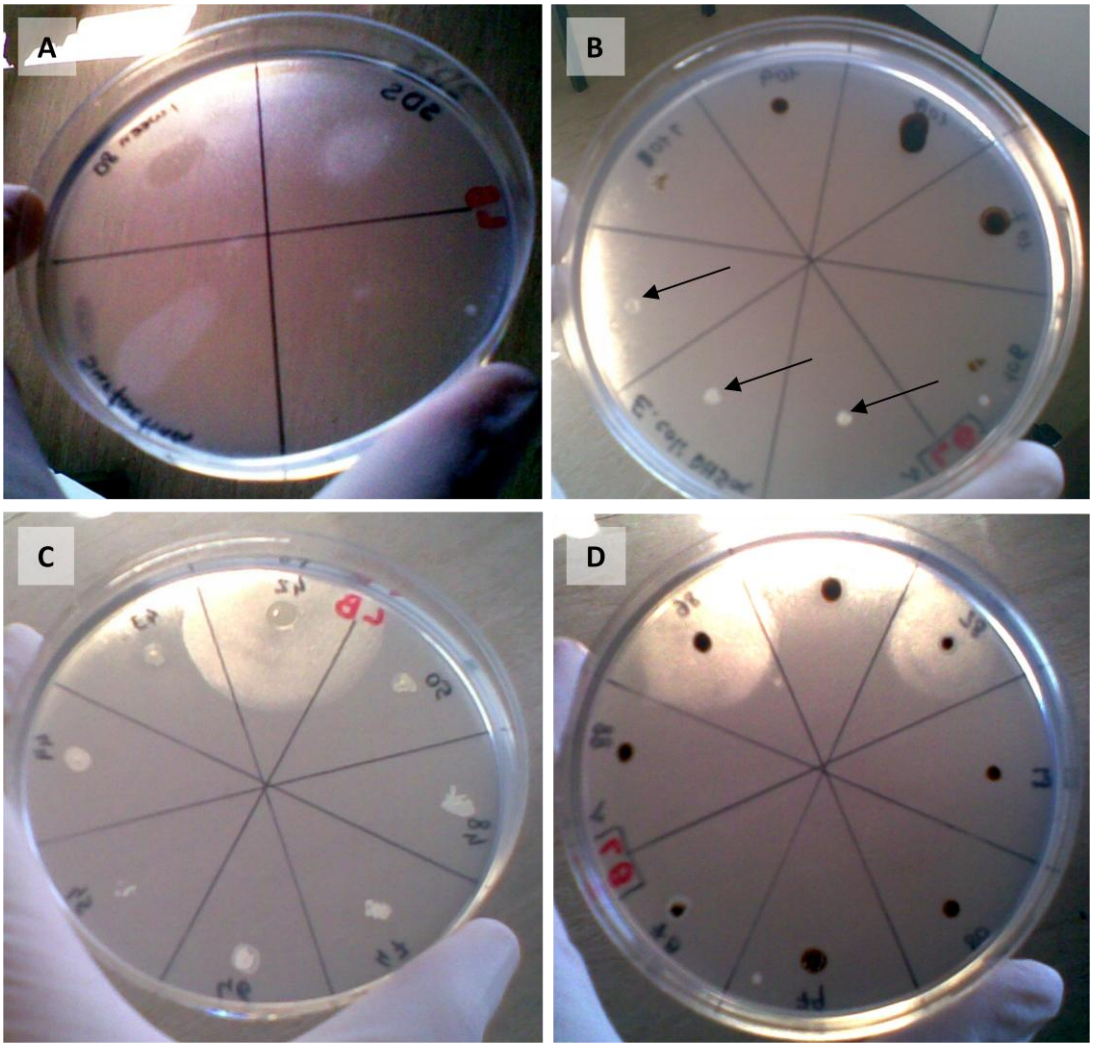

Figure 16 – Photographs of the results of the *atomized oil assay* used to test the production of biosurfactants by the isolates. A – Positive controls: left top corner, Tween 80 (0.008 mM); right top corner, SDS (10 mM); left bottom corner, surfactin (1.19 g L^{-1}). B – Negative control: colonies of *E. coli* DH5α marked with arrow. C – Example of positive result (halo) for one isolated strain cultivated in non-selective medium prior to the inoculation in LB medium for the atomized oil assay. D – Example of positive results (halos) for isolates cultivated in selective crude medium prior to the inoculation in LB medium for the atomized oil assay.

4. DISCUSSION

4. Discussion

4.1 Selective cultures with hydrocarbons as hydrophobic carbon sources

Hydrocarbons have shown good results when used as subtracts for the selection and isolation of biosurfactant producing microorganisms [119]. For this reason, petroleum hydrocarbons were chosen as substrates for the selective cultures, since the aim of this work was to isolate biosurfactant producing bacteria. Additionally petroleum hydrocarbons were also chosen, instead of other hydrophobic hydrocarbons, such as frying oils, so that these isolates could also have high potential in hydrocarbon remediation strategies.

After 2 months of incubation, the visual appearance of the selective cultures had changed from a biphasic suspension to a turbid culture medium without a clear separation between the hydrophobic and the hydrophilic phases. This was interpreted as a first indication of the presence of bioemulsifiers in the cultures, produced by the enriched bacterial populations, that reduced the interfacial tension between the two immiscible liquids [55]. Similar effects have been found in other hydrocarbon selective cultures with biosurfactant producing strains [120-122]. The emulsification effect has been interpreted as an increase of bioavailability of petroleum hydrocarbons which would be favorable to bioremediation strategies at sea [35].

The emulsification effect was particularly visible in the crude selective cultures and was very clearly observed in the isolation cultures in solid media. The emulsification of the hydrophobic substrate, that in this case was crude, may have caused the halo effect that was observed around isolated colonies. Also, some crude biodegradation may have occurred in the liquid selective cultures, as well as in the solid isolation cultures, because the hydrocarbon-rich substrate was provided as sole-carbon source selecting for hydrocarbonoclastic microbes.

4.2 Effect of the selective media on bacterial communities

4.2.1 Culturability

One of the objectives of this work was to select for the most suitable medium for the isolation of biosurfactant producing hydrocarbonoclastic bacteria from different environmental matrices. For that, a mineral medium was amended with one of 3 distinct carbon sources that were hydrophobic, selecting for biosurfactants producers, and rich in hydrocarbons, selecting for

microbes capable of using hydrocarbons as carbon sources. Fungi are a relevant component of the hydrocarbon-degrading microflora in the environment and to restrain their growth, cycloheximide, an inhibitor of protein biosynthesis in eukaryotic organisms, was added to the selective cultures. The colony counts (CFU) in non-selective media were used as an approach to determine the size of the community and not to the proportion of target organisms (hydrocarbonoclastic-biosurfactant producers) because the later may profit from the degradative activity of a few specialists, establishing complex syntrophic relations between community members[123]. Also, hydrocarbons can be toxic to non-degrading microorganism and the variation of the community size in the selective cultures ultimately reflects the balance between inhibitory and stimulatory effects of the different populations that compose each community [45]. The variation of the number of colony forming units from the third week to the end of the incubation, globally showed that the response of the culturable fraction depends on the origin of the inoculum and also on the carbon source provided.

On average, the sediment inoculum produced the highest CFU counts, and the maximum was observed in the selective medium containing crude. In diesel cultures, there was a steep decrease during the first 5 weeks and the counts remained low to the end of the incubation and in paraffin, counts were always low and stable. This may be related to the richness and diversity of carbon sources found in crude versus the less complex composition of diesel [4]. The cultures in which bulk sediment was used as inoculum also showed the highest average value for Shannon-Weaver index calculated from the DGGE profiles. Sediment bacterial communities are generally considered as highly diverse both in terms of community structure and physiological potential, with the capacity of using a wide range of carbon sources [124, 125].

Although rhizosphere microbial communities are usually considered to be more diverse than the communities from unvegetated sediments [106], in this case, they were probably more adapted to the use of root-derived carbon sources and could not use crude as efficiently as the more eclectic community from bulk sediments. The culturable fraction of the communities in the rhizosphere selective cultures was quite stable during the incubation period, with only a slight decrease of colony counts in the crude selective culture, towards the end of the incubation period indicating that these communities was less responsive to the selective pressure than bulk sediment or SML bacterial communities.

The SML micro-environment is considered to be enriched in hydrophobic substrates, namely some pollutants such as hydrocarbons [126]. Additionally, biosurfactant production is considered a widespread adaptation of bacterioneuston to the interfacial conditions of this

particular compartment of the water column [81]. After 3 weeks of incubation, the concentration of CFU in the cultures inoculated with SML water was still very high in the diesel and paraffin selective cultures. From the forth week on, there was a decrease in CFU in all the selective cultures using SML inocula but at the end of the incubation, the concentration of CFU was slightly higher in the paraffin selective cultures, in relation to the cultures with other carbon sources.

The general trend was a decrease in CFU in all selective cultures during the course of the incubation, which may be interpreted as a toxic effect or as a depletion of nutrients because of insufficient hydrocarbon utilization in relation to the carbon demand of the community. However, the least stressing conditions as inferred from the higher CFU values at the end of the incubation were obtained in paraffin medium for the SML and the rhizosphere inocula and in crude medium with the bulk sediment inoculum.

4.2.2 Structural diversity

The relative diversity of the bacterial communities developing in the different selective cultures at the end of the incubation was determined using the data obtained from the analysis of the DGGE profiles. Because the imposition of a strong selective pressure, such as the availability of a sole carbon source, could shape different communities in a convergent direction, the dendrogram was analyzed to check the closest (structurally more similar) communities. With the exception of all the paraffin amended cultures the crude-selective cultures of SML water and rhizosphere bacteria (CR and CA), there was not an enhanced similarity between communities forced to use the same carbon source. On the other hand, bulk sediment cultures with different carbon sources (DS and PS) were more similar between each other than with cultures initiated from different inocula but amended with the same carbon source. A similar effect occurred in the DR and PR cultures. The results show that paraffin has a stronger effect in the shaping of microbial communities and that this effect imposes on the initial community structure, in the sense that, different communities became more similar after selective incubation in paraffin medium. This may indicate an active adaptation of the community to a simpler carbon source. On the contrary, diesel and crude do not exert the same degree of control over the structure of sediment and rhizosphere bacterial communities. The SML community seems to be extremely responsive to the carbon source with very low similarity between communities challenged with different carbon sources (DA, PA and CA). Studies have shown that blooms of specialized bacteria tend to appear in contaminated areas of the SML [31], which could explain the low similarity between cultures of the SML with different carbon sources.

The estimated values for the Shannon-Weaver index indicate that the PA selective cultures were the more diverse and that DR was the least diverse. These results are in line with the information extracted from the analysis of the dendrogram and confirm the high physiological diversity of bacterioneuston and the susceptibility of rhizosphere communities to convergent adaptation and reduction of diversity in response to the carbon source. The fact that SML-paraffin selective culture exhibited the highest biodiversity is probably also the result of higher biodegradability of paraffin, comparatively to more complex hydrocarbons. Since the structure of paraffins consist in long open chains of exclusive single bonds, while other more complex hydrocarbons present in diesel and crude often have double bonds or cyclic structures making them much less biodegradable [4]. The average values (3 cultures) for the Shannon-Weaver index for each carbon source was higher for paraffin cultures (2.5331) than for crude (2.2509) or for diesel cultures (1.6726). This supports the hypothesis that paraffin is a more accessible substrate thus causing a smaller impact on the community diversity. The SML is generally considered as an extreme environment for microbial life from the physical and chemical perspectives where bacteria are adapted to respond to very sharp variations of environmental conditions [81]. The results may also indicate the higher degree of physiological redundancy of bacterioneuston, with different species adapted for the performance of similar ecological functions, in contrast with sediment bacterial communities in which a particular function may be conducted by a narrower range of physiological specialists.

The two communities with lowest Shannon-Weaver index correspond to the diesel selective cultures (DA and DR). This is probably due to the lack of diversity of petroleum compounds in the maritime diesel used, their low biodegradability, and the presence of some toxic molecules such as phenols and sulphur compounds [4, 127].

4.3 Hydrocarbonoclastic bacterial isolates

The fact that SML is a microenvironment where several kinds of chemical compounds, from surfactants to hydrophobic molecules accumulate [128], especially in semi-closed aquatic environments like estuarine systems and in close proximity to harbors and urban areas [83], makes it a prime place to find a high diversity of hydrocarbonoclastic bacteria. The main objective of this work was the search for biosurfactants producing bacteria but the option to use hydrocarbons as hydrophobic carbon sources also aimed the selection for hydrocarbon degrading bacteria.

By making a pre selection of isolates with different colony morphology and color, matrix of origin and carbon source used, we expected to obtain a set of the culturable fractions representative of the bacterial communities in each selective medium. After selection by such criteria, 111 isolates were cultivated and stored for further studies although only a sub-set of 66 isolates was actually typed by BOX-PCR for later DNA sequencing and screened for biosurfactants production. BOX-PCR results lead to a high number (58) of genetically different strains. Therefore it should be safe to assume that the estuarine system of Ria de Aveiro has a diverse community of potential biosurfactant producers and oil hydrocarbon degraders.

The largest subset of isolates was obtained from the crude selective cultures. This is probably due to the fact that crude oil is an extremely complex mixture of several kinds of hydrocarbons, and since each bacterial strain is only able to degrade up to two or three kinds of hydrocarbons compounds [22], a wider range of macroscopically different specialized strains is involved and a higher diversity of colonies was observed. Both the diesel and the liquid paraffin are much less complex mixtures with paraffin being much more easily degradable than some of the diesel compounds (some of them aromatic) due to its chemical structure.

The rhizosphere sediment was the inoculum that produced the larger number of macroscopically different colonies, rather than the SML water as one could anticipate from the values of the Shannon-Weaver index and because of the documented adaptation of bacterioneuston to elevated concentrations of hydrocarbons at the SML [81]. However because the selection of colonies was based on their macroscopic characteristics, it is possible that the genetic diversity of bacterioneuston was highly underestimated in the selection process.

Although the capacity for hydrocarbon degradation was not directly verified in the isolated strains, some of them were able to clear crude from the solid MSM plates. As previously referred, the clear zone around some of the colonies may be produced by processes of hydrocarbon solubilization by biosurfactants (removal and dispersion), hydrocarbon biodegradation (mineralization), or by a combination of both processes. Some of the isolates that produced halos in the MSM plates amended with crude tested positive for the production of biosurfactants by the atomized oil assay. However, the assessment of their potential for degradation of oil hydrocarbons still needs to be accessed.

4.4 Biosurfactant production

Of the initial set of 111 isolates, 66 were tested for the production of biosurfactants with the *atomized oil assay* [118], and from these, 17 (25.8 %) formed a halo surrounding the colonies characteristic of the presence of biosurfactant. The final number of 66 isolates tested resulted from the combination of the analysis of 32 isolates originated directly from selective pure cultures and 66 isolates from pure cultures in non-selective medium (TSB).

A subset of 32 of the isolates were tested from both the selective and non-selective media and the results confirm the influence of the culture medium on the production of biosurfactants as detected by the *atomized oil assay* conducted in LB colonies. Seven isolates were able to produce biosurfactants only when they had originated from the selective medium and not from TSA cultures. On the contrary, only 1 isolate was able to produce biosurfactant after cultivation in non-selective medium and not when tested directly from the selective medium. In 4 isolates biosurfactants production was detected in both selective and non-selective cultures and in 20 isolates, biosurfactants production was not detected. Some studies show that biosurfactant production can be enhanced by rich media [129, 130] while other studies use more complex carbon sources, such as PAHs [73, 131], which may explain the differences in biosurfactant production by the same isolates when transitionally cultivated in different media [132].

Similar percentual values of biosurfactants producing bacteria have been reported in the literature: 26.9 % from petroleum contaminated soil [122]; 23.8 % from hydrocarbon-contaminated environments [119]; 22.86 % in water samples collected from oil reservoirs [133]. However, different recovery percentages are often reported when using other carbon sources. The use of the chemical surfactants SDS and CTAB as sole carbon sources resulted in collection of surfactant-resistant bacterioneuston isolates from the Ria de Aveiro, 9.6 % of which were biosurfactants producers identified as Pseudomonads [134].

Overall, the environmental matrix from where the higher amount of biosurfactant producing bacteria were isolated was the SML, with 6 out of 15 (40.0 %) positive results. This result supports the fact that SML is a potentially rich source of biosurfactant-producing strains due to its particular physical-chemical properties. Bacterioneuston have to survive the surface tension of the air-water interface producing surface active compounds such as biosurfactants for that purpose. Also, the SML has structural analogies with biofilms and biosurfactant production is a common feature of biofilm organisms with important roles in the regulation of the tridimensional shape of the biofilm [135]. This also applies to rhizosphere bacteria that often form biofilms on plant root surfaces [136, 137]. The lowest percentage of biosurfactant producing

isolates was found in the sediment subset, with only 3 positive results in 26 (11.5 %). These results are not unexpected since there is little selective pressure in the sediment matrix *per se* regarding the production of biosurfactants. In a study by Viramontes-Ramos *et al.* (2010) [138] only 17 biosurfactant-producing isolates were found out of 324 (5,25 %) from samples of hydrocarbon-contaminated soils.

The percentage of biosurfactants producing isolates was highest among the subset isolated from the maritime diesel selective cultures, with 7 positive results out of 22 isolates tested (31.8 %). Maritime diesel is, of all the carbon sources tested, the most similar to the pollutants found in the proximity of the sampling site (due to the small harbors and leisure boats), therefore microbial communities may be more prepared to use it as a carbon source and use it to produce biosurfactants. Several bacterial strains have been isolated from diesel contaminated sites [139, 140]. Paraffin and crude oil selective cultures present the lowest proportions of biosurfactants producing isolates, both with 5 positive results in 24 (20.8 %) and 20 (25.0 %) isolates tested, respectively. However this value is not far from the other values detected in the selective cultures enriched with crude oil [122, 133].

In the overall analysis, the most successful combination for the isolation of biosurfactant producing bacteria was the bacterioneuston inoculum and diesel as hydrophobic carbon source. This combination produced the highest percentage of positive results with 4 positives out of 6 (66.7 %) isolates tested. Also, it corresponds to the selective culture with the second lowest genetic diversity, which can be an indication of natural adaptation of the community. The least successful combination was the sediment inoculum with crude as carbon source, because none of the isolates retrieved from this selective culture tested positive for biosurfactant production.

The metabolization of complex carbon sources such as hydrocarbons is often associated to the production of biosurfactants which in turn emulsify the hydrocarbons and enhance dispersal, making their transport into the intracellular compartment more easy and consequently accelerate biodegradation [141, 142]. In addition to the carbon sources, other growth conditions, such as temperature, pH, agitation, oxygen availability and nitrogen sources may influence the production of biosurfactants [61, 122]. It is possible that the conditions used in this work were still not optimal for the production of biosurfactants and biosurfactants production in isolates that tested negative with the atomized oil assay may not be completely ruled out. Some of the isolates may have produced small quantities of biosurfactant that were not detectable by the screening method used.

The results confirm that bacterioneuston communities represent a valuable seedbank for the prospection of microorganisms with interesting capacities from the biotechnological perspective and that is a worthy environment to explore in the search for efficient hydrocarbonoclastic biosurfactant-producers suitable for bioremediation approaches.

5.CONCLUSION

5. Conclusion

The main objective of this work was to assess the biotechnological potential of bacterial communities from different matrixes of the estuarine system of Ria de Aveiro regarding their capacity to produce biosurfactants and to degrade hydrophobic hydrocarbons. Those bacterial strains could be used in the future in bioremediation strategies of hydrocarbon contaminated coastal and estuarine sites. Crude oil, maritime diesel and liquid paraffin were the carbon sources used because they are common contaminants and highly hydrophobic. The environmental matrixes used as inoculants were SML water, rhizosphere bacteria and bulk sediment, which are recognized as highly diverse microbial communities and in the first two cases as natural sources of biosurfactant producing bacteria. In this work 9 selective cultures were obtained combining all carbon sources with all matrixes, so that a comprehensive study comparing the community size, diversity and frequency of culturable biosurfactant-producers of the different cultures could be made.

The findings of this study allow us to conclude that the all micro niches studied had bacterial communities with a high biotechnological potential for degradation of hydrophobic hydrocarbons and production of biosurfactants. The most suitable combination for the retrieval of biosurfactants producing bacteria was the use of diesel as carbon source and inoculum of SML water since it presented the highest percentage of biosurfactant producers (66.7 %), although it presented a low Shannon–Weaver diversity index when compared with the other selective cultures (1,5029). Bacterioneuston in particular, has shown great potential regarding the presence of biosurfactant producing potential hydrocarbonoclastic bacteria and community diversity, making it a prime place to isolate strains for future use in bioremediation strategies.

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