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PETROLEUM HYDROCARBONS BIOREMEDIATION IN A TEMPERATE SALT MARSH: PLANT–MICROORGANISMS INTERACTIONS

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Tese de doutoramento em Ciências Biomédicas

2013

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Petroleum Hydrocarbons Bioremediation in a Temperate Salt Marsh: Plant–Microorganisms Interactions

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Dedico este trabalho

Aos meus pais, Mário e Luisa.

À minha irmã e sobrinha, Rafaela e Mia.

À Núria.

À memória dos meus avós, Agostinho, Daniel e Leopoldina.

"I hear and I forget.

I see and I remember.

I do and I understand."

Confucius
(551 – 479 BC)

Agradecimentos

Quero deixar aqui registados os meus sinceros agradecimentos e profundo reconhecimento a todos aqueles que, directa ou indirectamente, contribuíram para a realização deste trabalho, nomeadamente:

Ao Professor Adriano Bordalo e Sá o meu profundo agradecimento pela orientação científica e conhecimentos que me proporcionou, pela disponibilidade e crítica ao longo de todo o trabalho em especial durante revisão final da Tese.

À Doutora Ana Paula Mucha agradeço a disponibilidade de co-orientar esta Tese, pela confiança depositada em mim no início desta minha jornada, pela dedicação e empenho com que me ajudou, pelas ideias e ajuda durante a realização da parte experimental do trabalho, pela disponibilidade e crítica ao longo de todo o trabalho.

À Doutora Marisa Almeida agradeço igualmente a disponibilidade de co-orientar esta Tese, pelas ideias, ajuda e crítica ao longo de todo o trabalho. Agradeço-lhe da mesma forma a oportunidade de usufruir das instalações e equipamento no laboratório Especiação Química e Biodisponibilidade (FCUP/CIIMAR), essencial para a realização deste trabalho.

À Fundação para a Ciência e Tecnologia, pelo apoio financeiro disponibilizado, através da bolsa de doutoramento (SFRH/BD/47631/2008) co-finaciada pelo POPH/FSE e pelo projecto Phytobio (PTDC/MAR/099140/2008), sem o qual a realização deste trabalho não teria sido possível.

Ao Centro de Interdisciplinar de Investigação Marinha e Ambiental da Universidade do Porto (CIIMAR-UP) e ao Instituto de Ciências Biomédicas Abel Salazar agradeço a utilização das instalações e equipamento, bem como o apoio logístico.

Ao Paulo Alves do Departamento de Biologia da FCUP agradeço a sua disponibilidade para identificar as plantas usadas neste trabalho.

Ao Nelson Matos agradeço a sua disponibilidade e ajuda em duas amostragens, bem como o companheirismo e amizade.

Aos elementos do laboratório de Hidrobiologia ICBAS/CIIMAR que acompanharam "*in loco*" esta aventura, nomeadamente a D. Lurdes Lima, Ana Machado, Catarina Café, C. Magalhães, C. Teixeira, Claudia Mendes, Eva Amorim, Isabel Azevedo, Izabela Reis, Liliana Carvalho, Maria Rovisco, Paula Salgado, Sandra Ramos e Tatiana agradeço a disponibilidade para ajudar nas tarefas, a amizade e o bom ambiente de trabalho. Em particular agradeço à Ana, C. Teixeira e Sandra pela transmissão de conhecimentos e métodos de trabalho.

Aos elementos do laboratório Especiação Química e Biodisponibilidade FCUP/CIIMAR, nomeadamente à Paula Guedes, Raquel Almeida e Jaqueline Cochofel agradeço a ajuda na determinação de TPH. Um agradecimento especial ao Moises Xavier pela grande ajuda na lavagem e preparação do material laboratorial. Ao Pedro Carvalho e Ana Cristina Rocha agradeço a disponibilidade para ajudar nas tarefas, a amizade e o bom ambiente de trabalho.

Ao Hugo, Olga e Ricardo do BOGA – CIIMAR agradeço o material e ferramentas que disponibilizam para a montagem das instalações experimentais.

Aos meus pais, à irmã e sobrinha, e restante família, e como é óbvio não existem palavras que descrevam o meu agradecimento à disponibilidade, ao apoio e ajuda que me deram ao longo da minha existência.

Um agradecimento muito especial à Núria pelos momentos partilhados no decorrer desta jornada.

Aos meus amigos, aos quais peço desculpa por não os referir pessoalmente, agradeço o convívio e apoio que ao longo da execução desta Tese me ajudou imenso a descontrair, de forma a enfrentar o árduo trabalho com um espírito revigorado.


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Resumo

Os estuários são, com frequência, considerados depósitos de contaminantes e, os sapais, considerados ecossistemas sensíveis com um importante papel ecológico, são particularmente vulneráveis à contaminação por hidrocarbonetos. Após a ocorrência de derrames de hidrocarbonetos, é importante limpar e recuperar essas áreas, o que pode ser uma tarefa complicada. O uso de plantas com microrganismos associados nas suas raízes, também conhecido como rizoremediação, poderá ser uma opção de mitigação economicamente viável e ambientalmente sustentável de contaminantes em solos e sedimentos. Até à data, não há conhecimento de investigação do uso das plantas de sapais europeus para a rizoremediação de hidrocarbonetos em ambiente estuarino. Assim sendo, o objectivo deste estudo foi avaliar o potencial de diferentes espécies de plantas normalmente existentes em estuários Europeus temperados, como o estuário do rio Lima, para mitigar a contaminação por hidrocarbonetos em sedimentos.

Em primeiro lugar foram recolhidos sedimentos não colonizados e colonizados (rizosedimentos) por diferentes plantas (*Juncus maritimus*, *Phragmites australis*, *Triglochin striata* e *Spartina patens*). Os quatro locais de amostragem localizaram-se na parte inferior e intermédia do estuário do rio Lima, sendo as amostras obtidas durante um ciclo de vida das plantas. Os sedimentos foram caracterizados em termos físico-químicos, contagem total de células (TCC), abundância de microrganismos degradadores de hidrocarbonetos (HD) e concentração de hidrocarbonetos totais de petróleo (TPH). Adicionalmente, o potencial de degradação de hidrocarbonetos foi avaliado quer através de diferentes experiências laboratoriais quer em estufa, neste caso com a duração de cinco meses, em condições que simulavam o ambiente estuarino. Nestas experiências foram testados diferentes tratamentos de bioremediação: rizoremediação, bioestimulação (adição de nutrientes para a promover a actividade dos degradadores indígenas) e bioaumento (adição de microrganismos degradadores).

Os resultados obtidos neste estudo mostraram que as plantas de sapal influenciam as comunidades microbianas, aumentando a abundância microbiana total e as populações de HD na sua rizosfera. Verificou-se também

que plantas diferentes têm influência distinta na dinâmica das populações de HD, que por sua vez parecem ser marcadamente favorecidas por plantas com raiz fasciculada (*P. australis* e *T. striata*), mas não no caso de plantas com raiz adventícias (*J. maritimus*). Esta influência foi especialmente acentuada nas épocas de maior actividade da planta. Adicionalmente, as características naturais dos sedimentos podem também influenciar a composição bacteriana da rizosfera, bem como a distribuição e biodisponibilidade dos hidrocarbonetos e, portanto, condicionar o potencial de degradação de hidrocarbonetos pelos microrganismos. Nas experiências com contaminação por hidrocarnonetos observou-se que o principal factor determinante para a estrutura da comunidade bacteriana nos sedimentos foi a presença e a espécie de planta, superando a influência do contaminante assim como da adição de nutrientes destinado a potenciar a actividade microbiana. No entanto, as distintas comunidades microbianas responderam da mesma forma à contaminação com (i) aumento da abundancia, (ii) mudanças na estrutura, e (iii) redução da diversidade.

As experiências em laboratório e em estufa mostraram que as comunidades microbianas associadas às raízes de *J. maritimus* e *P. australis* apresentaram potencial para degradar hidrocarbonetos em sapais. De referir que nos estudos em estufa, a degradação de hidrocarbonetos em sedimentos não colonizados foi insignificante, mesmo com a aplicação de tratamentos de bioremediação (bioestímulo e bioaumento). Os tratamentos de bioremediação também não aumentaram o potencial da rizoremediação de hidrocarbonetos. Este resultado indica que os nutrientes podem não ser o principal factor que afecta a degradação de hidrocarbonetos em sapais. Os estudos efectuados chamaram a atenção para a especificidade de cada espécie de planta em termos de necessidades nutricionais para a rizoremediação. De facto, a adição de nutrientes pode, inclusivamente reduzir a eficiência da interacção entre planta-microrganismos em termos de degradação de hidrocarbonetos devido a um efeito negativo do aumento de nutrientes no desenvolvimento do sistema radicular.

Estes resultados contribuem para a compreensão dos mecanismos que influenciam a mitigação de hidrocarbonetos pelas associações de planta-microrganismos e, portanto, devem ser tidos em consideração aquando do desenho de estratégias de rizoremediação em estuários.

Abstract

Estuaries are often considered sinks for contaminants, whereas salt marshes emerge as sensitive ecosystems that serve many ecological functions, and extremely vulnerable to hydrocarbon contamination. After major or minor oil spills, it is important to clean and recover these areas, which can be a difficult task. The use of plants and microorganisms associated to their roots, referred as rhizoremediation, can be an economically feasible and environmentally sustainable cleanup option to breakdown contaminants in soil and sediments. To date, European salt marsh plants have not been assessed for their hydrocarbon rhizoremediation potential in estuarine environments. In this vein the aim of the present study was to evaluate the potential of salt marsh plant species usually found in the temperate European estuaries, such as the Lima River estuary, for the remediation of petroleum hydrocarbons in sediments.

Firstly, sediments un-colonized and colonized (rhizosediments) by different plants (*Juncus maritimus*, *Phragmites australis*, *Triglochin striata* and *Spartina patens*) were sampled in four sites at the lower and middle estuary throughout the plants phenological cycle. Sediments were characterized in terms of physical-chemical characteristics, total cell counts (TCC), hydrocarbon degraders (HD) abundance and total petroleum hydrocarbons (TPH) concentrations. Afterwards, evaluation of hydrocarbon degradation potential was assessed in fortnight laboratory experiments, and in a 5-month greenhouse experiment (with environmental conditions similar to those found at the estuary), where different bioremediation treatments: rhizoremediation, biostimulation (*e.g.* the injection of nutrients to induce microbial propagation of the native microbial population), and bioaugmentation (*i.e.* the addition of enriched microbial cultures, resistant to the pollutant, to enhance its degradation) were tested.

Results of this research showed that salt marsh plants influence the microbial community, by fostering the development specific HD populations and increasing TCC in its rhizosphere. Distinct plants have different influence on the dynamics of HD populations, which seemed to be markedly thriving in the rhizosphere of plants with fibrous root morphology (*P. australis* and *T. striata*) but to a less extend in the case of plants with adventitious roots (*J. maritimus*).

These outcomes are especially highlighted in seasons of higher plant activity. Moreover, natural sediment characteristic influenced rhizosphere bacterial composition as well as the distribution and bioavailability of hydrocarbons, with consequences for the rhizosphere microbial potential for hydrocarbon degradation.

When exposed to petroleum contamination, the presence of the plant and the plant species emerged as the major factor for shaping the bacterial community structure, overriding the petroleum and nutrients influence. Nevertheless, distinct salt marsh microbial communities responded similarly to petroleum contamination by (i) increasing their abundance, (ii) changing their structure, and (iii) decreasing their diversity.

Laboratory and greenhouse experiments indicated that microbial communities associated to *J. maritimus* and *P. australis* roots had a potential to degrade petroleum hydrocarbons in a salt marsh environment. It should be noted that in greenhouse studies, hydrocarbon degradation in un-colonized sediments was negligible regardless the bioremediation treatments (biostimulation and bioaugmentation) applied. Bioremediation treatments did not enhance the TPH rhizoremediation potential. This results points that nutrients may not be the main limiting factor affecting PHC degradation in salt marshes. These studies also alerts to the specificity of each plant species in terms of nutritional requirements for rhizoremediation, and that nutrient addition can decrease the hydrocarbon degrading ability of the plant-microorganism association, due to a counter-effect on the root system development.

These findings can assist our understanding on the mechanisms that influence hydrocarbons remediation by plant-microrganisms association, and should be considered when designing rhizoremediation strategies in estuaries.

List of papers

The author of this thesis states that he gave a major contribution to the conceptual design and technical execution of the experimental work that originated the presented results.

The following manuscripts published or submitted were used in this PhD thesis:

1. **Ribeiro H**, Mucha AP, Almeida CMR, Bordalo A (2011). Hydrocarbon degradation potential of salt marsh plant-microorganisms associations. Biodegradation 22, 729-739.

2. **Ribeiro H**, Almeida CMR, Mucha AP, Teixeira C, Bordalo A (2012). Influence of natural rhizosediments characteristics on hydrocarbons degradation potential of microorganisms associated to *Juncus maritimus* roots. International Biodeterioration and Biodegradation http://dx.doi.org/10.1016/j.ibiod.2012.05.039

3. **Ribeiro H**, Almeida CMR, Mucha AP, Bordalo A (2013a). Influence of different salt marsh plants on hydrocarbon degrading microorganisms abundance throughout a phenological cycle. International Journal of Phytoremediation 15, 715-728.

4. **Ribeiro H**, Mucha AP, Almeida CMR, Bordalo A (2013b). Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh. Science of Total Environment 458-460, 568–576.

5. **Ribeiro H**, Almeida CMR, Mucha AP, Bordalo A (2013c). Potential of salt marsh plants for the removal of petroleum hydrocarbon: rhizoremediation, biostimulation or bioaugmentation? Submitted to Journal of Hazardous Materials

Contents

Abbreviations

AFL	ARISA fragment length
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ARISA	Automated rRNA intergenic spacer analysis
BA	Bioaugmentation
BH	Bushnell Haas
BS	Biostimulation
DAPI	4',6'-diamidino-2-phenylindole
EC	Electrical conductivity
FITR	Fourier transform infrared spectrophotometry
GC-FID	Gas chromatography-flame ionization detection
HD	Hydrocarbon-degrading microorganisms
IGS	Intergenic spacer
INT	Iodonitrotetrazolium violet
L1	Sampling site 1
L2	Sampling site 2
L3	Sampling site 3
L4	Sampling site 4
MPN	Most probable number
MDS	Multidimensional scaling
NA	Natural attenuation
OM	Organic matter
OTU	Operational taxonomic units
PAH	Polycyclic aromatic hydrocarbons
PC	Principal component

PCA	Principal components analysis
Pet.	Petroleum
PHC	Petroleum hydrocarbons
RR	Rhizoremediation
T_0	Time zero days
T_{15}	Time fifteen days
T_{150}	Time one hundred fifty days
TCC	Total cell counts
TPH	Total petroleum hydrocarbons

Chapter 1

General introduction

General introduction

1.1 Estuaries

The term “estuary” has historically referred to the lower tidal reaches of a river. In fact, the word "estuary" is derived from the Latin word *aestuarium,* meaning marsh, which in itself is derived from the term *aestus*, meaning tide (Elliott and McLusky, 2002). There have been many definitions proposed to describe an estuary, most of them suggested according to geomorphology, hydrography and biological features. Pritchard (1967) described an estuary as "a semi-enclosed coastal body of water, which has a free connection with the open sea, and within, sea water is measurably diluted with freshwater derived from land drainage". He further divided estuaries into four classes based on physical characteristics: (i) Drowned river valley, (ii) Fjord, (iii) Bar-built, and (iv) Tectonic. However, the definition did not account for the influence of tide and excluded a number of coastal water bodies such as coastal lagoons and brackish seas. Intuitively, it seems that an estuary could be defined most simply based on salinity, specifically as an area where, due to the effect of tides, water is neither truly saline (>30 g L^{-1}) nor truly fresh (0 g L^{-1}) (Chapman and Wang, 2001). Dyer (1997) suggested a definition based on the physical definition of Pritchard but including a mention on tidal influences: “An estuary is a semi-enclosed coastal body of water which has a free connection with the open sea, extending into the river as far as the limit of tidal influence, and within which sea water is measurably diluted with freshwater derived from land drainage". In addition to physical characteristics, there are effectively four different types of estuaries based on the tidal amplitude, and the relationship and mixing between fresh and salt water: (i) highly stratified or salt wedge; (ii) Fjords; (iii) Shallow, partially mixed; and (iv) Vertically homogeneous estuaries (McLusky and Elliott, 2004).

According to McLusky and Elliott (2004), salinity at any particular point of an estuary depends on the relationship between the volume of tidal seawater and the volume of freshwater entering the estuary, as well as the tidal amplitude and the topography of the estuary, but in general it is possible to recognize various divisions or classifications within an estuary (Table 1.1).

Table 1.1 - Classification of estuarine divisions (source: McLusky and Elliott, 2004). Salinity is defined according to the Practical Salinity Scale.

Division	Tidal	salinity	Venice system classification
River	Non-tidal	< 0.5	Limnetic
Head	The highest point reached by tides		
Tidal fresh	Tidal	< 0.5	Limnetic
Upper	Tidal	0.5 - 5	Oligohaline
Inner	Tidal	5 - 18	Mesohaline
Middle	Tidal	18 – 25	Polyhaline
Lower	Tidal	25 – 30	Polyhaline
Mouth	Tidal	> 30	Euhaline

An estuary is a dynamic ecosystem providing not a simple habitat, but rather a multiple, complex and interrelated web of habitats. The connection with the open sea through which the sea water enters with the rhythm of the tides, blending with fresh waters, provide strong gradients in many physical and chemical variables (*e.g.* salinity, temperature, pH, dissolved oxygen, nutrients and composition of particles), not only along their length, from river to sea, but also laterally and vertically (water columm). These abiotic gradients are important in determining the nature of estuarine habitats, particularly influences the adapted assemblages of organisms in each different habitat. An important vertical gradient in the aquatic system is the lighted zone, called the *euphotic* zone, while the zone with no light is the aphotic zone (Day et al., 2012). If light reaches the bottom, rooted plants and plankton can generally photosynthesize and live attached to the bottom. Tidal waters are also an important vertical gradient of estuaries that particularly influence the intertidal zone, which is alternately flooded and exposed, supporting unique communities of plants and animals, especially adapted for life at the margin of the sea (McLusky and Elliott, 2004). In the intertidal zone there may be found habitats with great ecological value, including un-vegetated and vegetated habitats such as sand; mudflats; salt marsh or mangrove wetlands; reefs of oysters; and algal beds (Day et al., 2012).

Salt Marshes

A salt marsh, also known as tidal marsh, is a coastal ecosystem in the intertidal zone between land and open salt water or brackish water that is regularly flooded by the tides. Salt marshes are located in mid and high latitudes regions worldwide, while mangroves are located in subtropical and tropical zones. Several types of marsh are recognized (Figure 1.1) based on their physical and geomorphological settings. Although many salt marshes are estuarine, they can also be found associated with barrier islands, spits, embayments and open shores exposed to low wave energy, as well as fringing coastal lagoons (Allen, 2000).

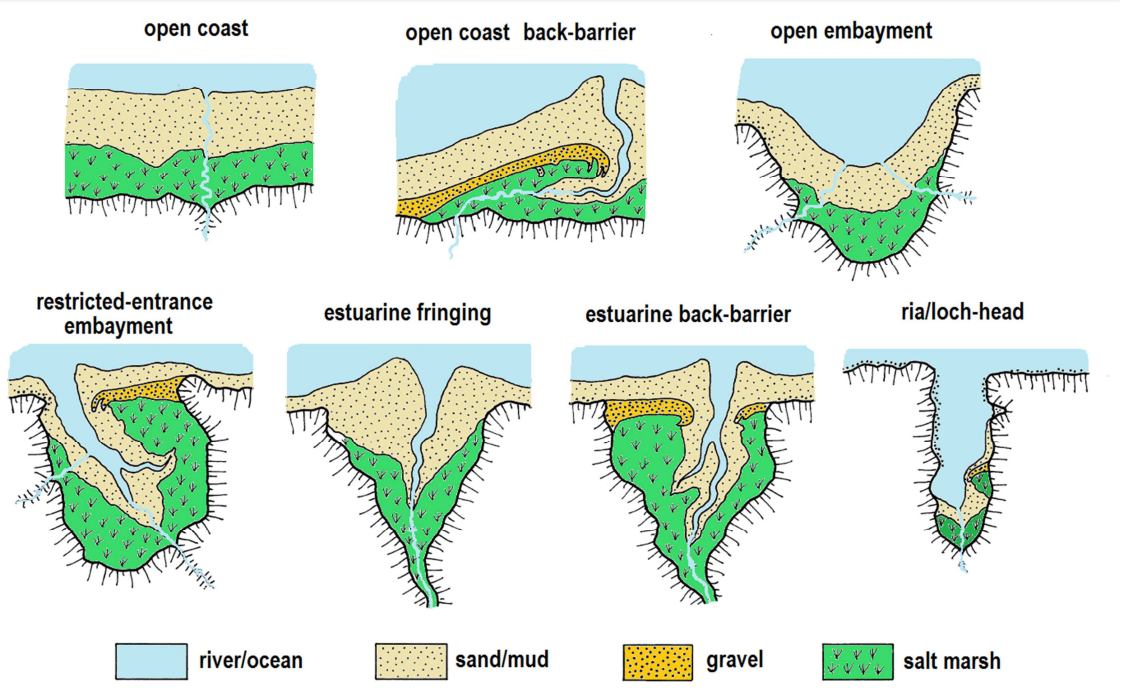

Figure 1.1 - A geomorphological classification of salt marshes (adapted from Allen, 2000).

Salt marshes are characterized by the alluvial sediments deposited on the shore and colonized by dense stands of salt-tolerant plants such as herbs, grasses or low shrubs adapted to complete their lifecycle in salty environments (Kennish, 2001). The differentiation between plants and/or associations among salt marshes can be explained with both ecological and biogeographic factors. Moreover, the spatial distribution of halophytic vegetation over salt marshes is

not random or spatially uncorrelated but is, on the contrary, organized in a characteristic zonation (Silvestri et al., 2005). This zonation is based on the topography and characteristic plant assemblages, and are classified as lower, middle and upper marsh (*e.g.* Figure 1.2), according to the number of tidal submergences per year (Adam, 1990). Plants growing toward the landward side of the marsh tend to be less tolerant of salt or brackish water. The floristic composition and structure of these zones is sufficient to distinguish from each other, and reflects the behavior of a few dominant species. At a finer scale, there is also spatial variation in species occurrence related to microtopography; and thus many plant species are distributed, not in discrete bands (zones), but in mosaics (Adam, 2002).

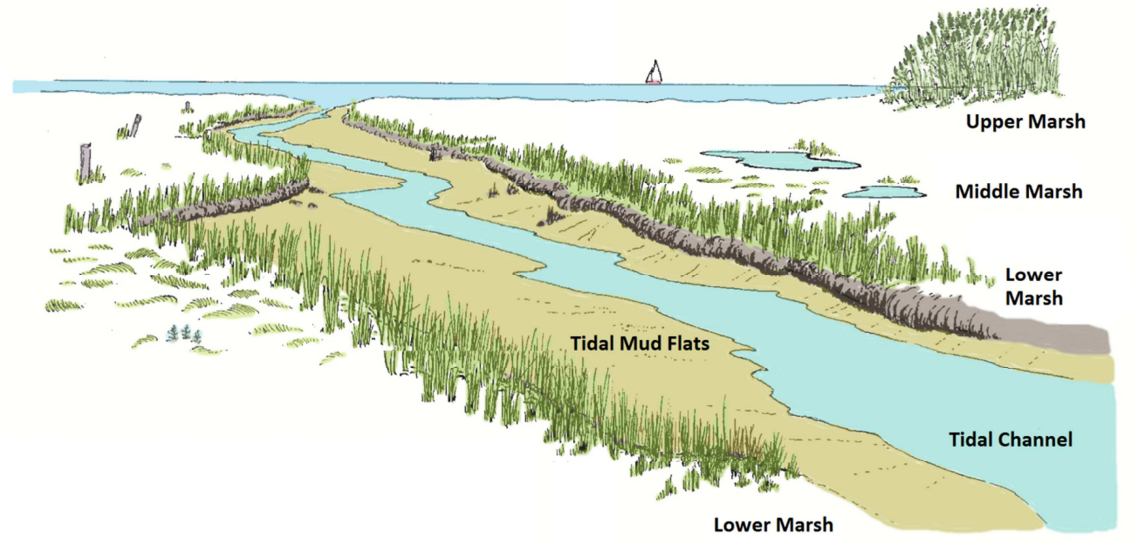

Figure 1.2 - Typical salt marsh zonation: upper, middle and lower marsh (modified from http://www.outerisland.org/OI_media-resources/OI_ecology/OI_diagrams-sketches/NEAQ-guide-images/saltmarsh-sketchdiagram-color.jpg).

The appreciation of the importance of salt marshes has been brought even more sharply into focus because of the threats posed by predicted rises in sea level as a result of global climatic change (Boorman, 1999). Salt marsh is classified as one of the most productive coastal ecosystems on Earth (Costanza et al., 1997), since primary productivity is often extraordinary high in comparison to the open sea or coastal waters (McLusky and Elliott, 2004). High rates of primary production within estuaries are generally associated with nutrient loadings from freshwater input. Salt marshes have a whole range of

functions. Such as a role as sediment trap; regulate the water quality and contribute to stabilize the coastlines; may remove the excess of nutrients, toxic chemicals and disease-causing organisms.

The value of saltmarshes for wildlife conservation has been also recognized for many years, particularly in terms of the wide variety of plants and animals associated with them. The creeks of tidal marshes also provide spawning sites and nursery areas for many fish species; and also provide feeding, roosting and nesting areas for a wide range of bird species (Boorman, 2003).

1.2 Salt marsh contamination

Since the earliest civilizations, estuaries have been recognized as an important place for human settlement due their prime location. Estuaries provide a unique access to both land and sea. Therefore, many large cities are located in theirs banks. Human populations have a long history of exploiting the coastal and estuarine areas for food, transport, commerce and for settlement (Costa-Dias et al., 2010). As an inevitable consequence of human population growth, contamination of estuaries and coastal waters will continue to suffer from the legacy of past poor planning and lack of regulation (Adam, 2002). Estuaries are the eventual repository for contaminants that are discharged directly or indirectly into these environments or upstream and delivered by the rivers and streams that feed them. There are many forms of contamination as well as other types of stress on the system resulting from human uses. Indeed, human activities resulting in the insertion of xenobiotics and toxins, energy change, overstimulation of biota, physical structures, and the input of nonindigenous organisms can change estuaries functioning (McLusky and Elliott, 2004). Contaminants can be categorized in trace metals, synthetic organic compounds, hydrocarbons, radioactivity, inert (physical) materials, nutrient, and organic matter (OM). Some of this contamination is unplanned or accidental; nevertheless, non-point-source pollutants are likely to remain a major problem in both developed and developing countries (Panda and Behera, 2003). Nonpoint source contamination generally results from land runoff, atmospheric deposition, drainage, seepage or hydrologic modification,

ultimately find their way into groundwater or small watercourses, and finally to estuaries in the form of sediment and chemical loads carried by rivers.

Petroleum hydrocarbon pollution

In the last decades, levels of petrochemical products in the environment, particularly in estuaries and coastal areas have increased (Lima et al., 2007). Crude oil or petroleum may be characterized in four primary fractions, namely the aliphatic hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. Resins and asphaltenes consist of non-hydrocarbon polar compounds, with trace amounts of nitrogen, sulfur and/or oxygen in addition to carbon and hydrogen, and often forming complexes with heavy metals. Both are important in the quality analysis of crude oil. Light oils are typically high in saturated and aromatic hydrocarbons, with a smaller proportion of resins and asphaltenes, and heavy oils have a much lower content of saturated and aromatic hydrocarbons and a higher proportion of the more polar chemicals (Head et al., 2006). Petroleum hydrocarbons (PHC) are a complex mixture of hydrocarbons, varying widely in both physical and chemical properties depending on the source (Leahy and Colwell, 1990). The principal constituents of PHC are the elements hydrogen (10-14%) and carbon (83-87%).

Occurring in varied structural configurations, PHC can be broadly divided into 2 families: aliphatic and aromatic (Figure 1.3). The aliphatic are further divided into 4 classes: alkanes, alkenes, alkynes and cycloalkanes. Alkanes, alkenes, alkynes are hydrocarbons built with straight or branched chains. Alkanes are saturated, whereas alkenes and alkynes are unsaturated hydrocarbons due to the presence of double and triple bonds between two carbons, respectively. The cycloalkanes present one or more saturated rings. The structural components of aromatic hydrocarbon molecules are one or more 6-membered carbon rings (benzene) that demonstrate high chemical stability due to double bonds. The family is divided into monoaromatics and polycyclic aromatic hydrocarbons (PAH). Monoaromatics have one ring such as benzene, toluene, ethylbenzene and xylene, collectively known as BTEX. PAH are condensed aromatic ring structures with 2 or more benzene rings.

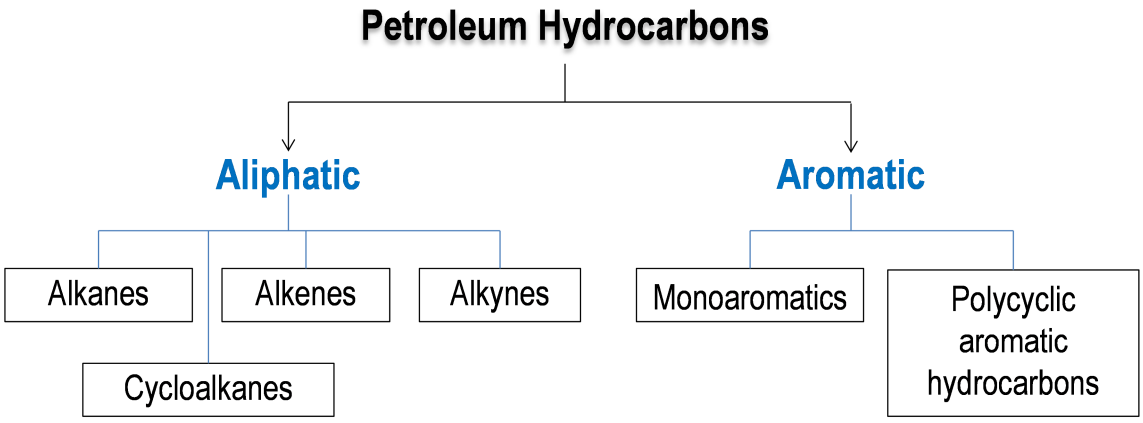

Figure 1.3 - Petroleum hydrocarbon structural relationships.

Saltmarshes may become contaminated by PHC in a number of ways; from municipal and industrial wastewater discharges, chronically by frequent small leakages and spills from commercial ships and recreational boating, urban runoff from land-based traffic accidents, major shipping accidents, and from offshore production facilities. Of these, major oil spill accidents have drawn great attention due to their catastrophic impact on the coastal environment, a number of which have affected saltmarshes, such as the recent one, Deepwater Horizon 2010, in the Gulf of Mexico (Natter et al., 2012). The degree of oil impact also depends on various factors, such as the extent of oil coverage, the amount and type of oil the plant species, the season of the spill, the soil composition and on climate (Beazley et al., 2012; Zhu et al., 2004). Moreover, salt marshes are especially vulnerable to oil spills because the inherently low wave energy of a wetland does not physically remove oil effectively.

Oil spills have been known to cause acute and long-term damage to salt marshes (Lin and Mendelssohn, 1996). These impacts include reduction in population and growth rate or abnormal growth and regrowth of plants after initial impact. Different wetland plants also respond differently to oil spills, particularly to crude oil heavy contamination that can lead to widespread mortality, and plants may require a decade or more to recover (Lin and Mendelssohn, 2012; Vega et al., 2009). Plants are more sensitive to oiling during the growing season than other periods (Pezeshki et al., 2000). The sediment type also plays an important role. In general, oil remains longer in

soils with higher OM and, therefore, has greater impact on resident plants (Otten et al., 1997).

Oil spills on coastal wetlands not only damage plants but also have serious consequences for other organisms such as zooplankton and benthic invertebrates. Seabirds that congregate on the salt marshes suffer from the destruction of their feeding grounds (Zhu et al., 2004). Fish are also contaminated, but to a lesser extent (Teal and Howarth, 1984). These impacts include obvious immediate consequences, such as widespread animal mortality due to smothering and toxic effects, and more subtle long-term effects. Oil can also change animal's feeding and reproductive behaviors. The extent of the impacts also depends on many factors, such as the life cycle and the life habit of organisms, and the duration of oil exposure (Zhu et al., 2004).

Considering the different sensitivity of salt marsh species and populations to oil spills, the effects of oil on salt marsh ecosystems and recovery still require further investigation. Recovery times vary from a few years to over a decade, and in a few extreme cases, salt marsh ecosystems have not fully recovered decades after the initial oil spills (Oudot and Chaillan, 2010; Teal et al., 1992; Vega et al., 2009).

1.3 Mechanisms for remediation of hydrocarbons

Risk assessment for environmental pollution is currently focused on estimating human exposure (Brassington et al., 2007). However, very little attention is given to the ecological risks of soil/sediment contamination. Remediation of PHC can be a challenge, and there are many factors that affect decision-making during the remediation process. Financial constraints, regulatory and civic pressures, risks, liability and environmental conditions all contribute to the choice of remediation strategies (Filler et al., 2009).

Since PHC contamination can cause serious damage to salt marsh ecosystems, which possess very high ecological importance for numerous biota species, effective countermeasures are essential to minimize these ecological impacts. Considering the characteristics of salt marsh ecosystems, conventional techniques used for remediation of hydrocarbon contaminated soil have

enormous drawbacks. Physical and/or chemical treatment, *in situ* as well as *ex situ* remediation, used to destroy (*i.e.* chemically convert), separate, or immobilize the contamination comprises strategies such as: i) mechanical oil removal by stripping surface sediments; ii) high/low pressure or hot water flushing; iii) dispersants and chemical oxidation; iv) soil vapor extraction; v) electrokinetic separation; vi) solidification/stabilization; vii) chemical extraction; vii) dehalogenation; ix) sediment washing; x) incineration; and xi) thermal desorption. Although *ex situ* treatment requires shorter time periods than *in situ* treatment; this strategy is very difficult to implement. It increases engineering equipment and costs, creating potential threats to human health and the environment that can arise during handling and transport hazardous material (Vidali, 2001). Indeed, these physical and/or chemical treatments *in situ* or *ex situ* have been reported to be more harmful than successful in the restoration of salt marshes. In one hand, they can be very effective at reducing levels of a range of contaminants; but on the other, the significant enormous environmental damage, the high costs involved, as well as the lack of public acceptance, especially due to the major disruption of normal activities, claims for a more economic and environmentally friendly remediation approaches, such as natural biological processes.

1.3.1 Hydrocarbon-degrading microorganisms

All hydrocarbon biological remediation processes have a shared principle, a technology based in specialized hydrocarbon-degrading microorganisms (HD). It is well known that HD are ubiquitous in the environment, and have been found in habitats ranging from polar soils (Whyte et al., 2002) to marine environments (Yakimov et al., 2007), including salt marshes (Daane et al., 2001). While these degraders are generally found at much lower concentrations in pristine environments (Margesin et al., 2003), the presence of hydrocarbons, likely due to atmospheric deposition of pyrogenic hydrocarbons, has resulted in the maintenance of degradation potential within populations of un-contaminated sites.

Isolating and identifying microorganisms responsible for hydrocarbon transformations worldwide have long been recognized as important for obtaining the most promising strains for site decontamination (Bu□ková et al.,

2013). A contemporary review lists 79 bacterial genera that can use hydrocarbons as a sole source of carbon and energy, as well as 9 cyanobacterial genera, 103 fungal genera and 14 algal genera that are known to degrade or transform hydrocarbons (Head et al., 2006). The phylogenetic diversity of HD is vast, and several recurrent groups are found in both marine and soil/sediment environment. *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Arthrobacter*, *Flavobacterium*, *Corynebacterium*, *Rhodococcus*, *Ralstonia*, *Stenotrophomonas*, *Acinetobacter*, *Mycobacterium*, *Micrococcus*, *Alcaligenes*, and *Nocardioides* species are all hydrocarbon degraders regularly isolated (Aislabie et al., 2008; Fritsche and Hofrichter, 2000; Hamamura et al., 2006; Juck et al., 2000; Kästner et al., 1994; Liste and Prutz, 2006; Rosenberg, 2006).There is some evidence that groups with a high rate of reproduction (r-strategist) become more prevalent in initial response to hydrocarbon contamination (*e.g.* Beazley et al., 2012; Kostka et al., 2011; Margesin et al., 2003).

These studies found that phyla containing previously described hydrocarbon-degrading bacteria (*Proteobacteria*) increased in relative prevalence in contaminated soils/sediments compared to pristine environments. The dominance of a given degrader group may be a function of time. In fact, other groups of bacteria (*e.g. Actinobacteria*) that optimize the utilization of environmental resources (k-strategists) tend to be more successful in resource-limited situations, such as the more recalcitrant fuel components (Margesin et al., 2003). Beazley et al. (2012) found, during the months of oil inundation from the Deepwater Horizon oil spill into salt marsh, a significant increase of the relative richness and abundance of phyla containing previously described HD (*Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*). Once hydrocarbons were below detection, the relative richness and abundance of these phyla decreased; except for the case of *Firmicutes,* that continued to increase in relative richness and abundance even when hydrocarbon concentrations were below detection.

Numerous studies have found that while hydrocarbon contamination does shift microbial populations, community responses differ with soil/sediment type (*e.g.* Hamamura et al., 2006). Other studies however, have shown that plant species alter the general phylogenetic community structure of rhizosphere bacterial communities (*e.g.* Grayston et al., 1998; Marschner et al., 2001), a

fact that may condition microbial response to hydrocarbons. Nevertheless more research is need to understand the mechanisms that regulate the plant-microbial interactions in general and their influence on the HD population.

Hydrocarbon metabolism

Microorganisms seem to be able to degrade and utilize practically any hydrocarbon as a carbon and energy source, and associated metabolic reactions have been studied intensively for decades (Robertson et al., 2007; Van Hamme et al., 2003). Bacterial communities degrade these hydrocarbons via numerous different catabolic pathways (van Beilen and Funhoff, 2007) which are subject to numerous controlling mechanisms. The metabolic pathways (Figure 1.4) that HD uses can be either aerobic (*i.e.* they utilize oxygen as the primary electron acceptor) or anaerobic (*i.e.* they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation (McNally et al., 1999). One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction.

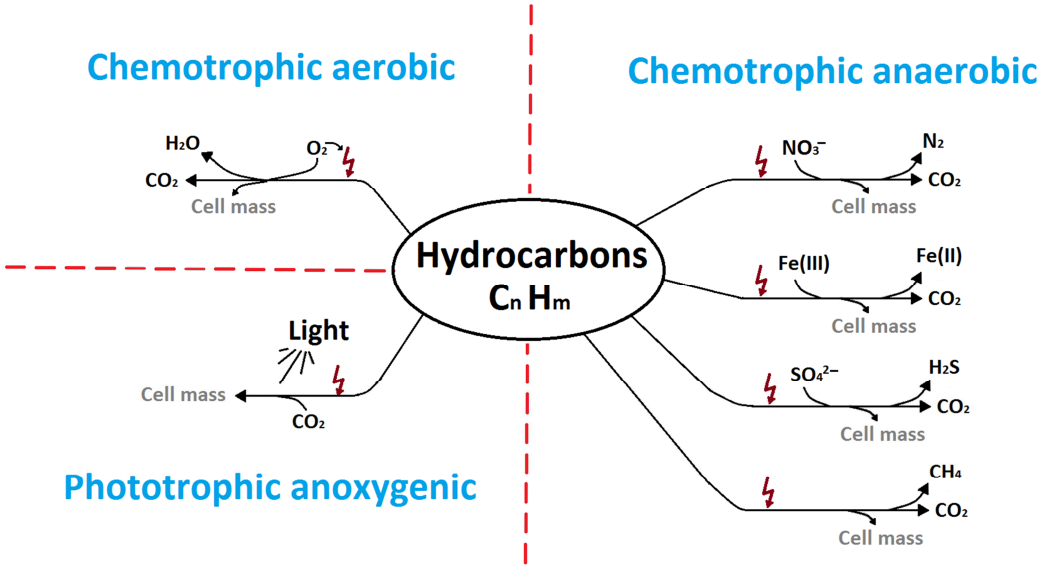

Figure 1.4 - Hydrocarbon metabolic pathways proposed to microorganisms (modified from Widdel and Rabus, 2001). Jagged arrows indicate hydrocarbon activation.

According to Fritsche and Hofrichter (2000) the essential characteristics of aerobic hydrocarbon degradation by bacteria are (Figure 1.5):

(i) Metabolic processes for optimizing the contact between the microbial cells and hydrocarbons.

(ii) The initial intracellular attack of organic pollutants is an oxidative process, the activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases.

(iii) Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, *e.g.* the tricarboxylic acid cycle.

(iv) Biosynthesis of cell biomass from the central precursor metabolites, *e.g.* acetyl- CoA, succinate, pyruvate. Sugars required for various biosyntheses and growth must be synthesized by gluconeogenesis.

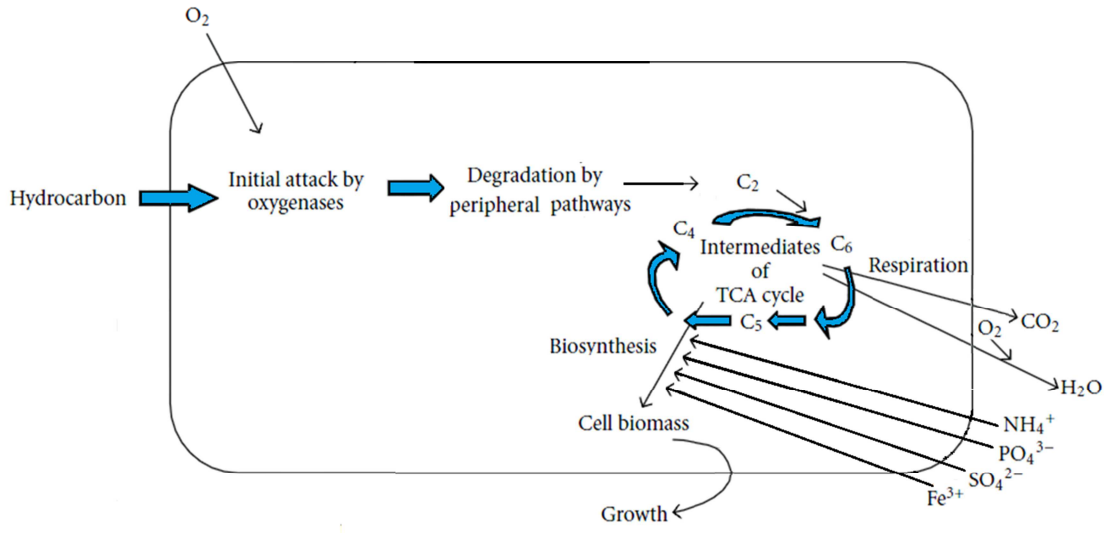

Figure 1.5 - Main principle of aerobic degradation of hydrocarbons: growth associated processes (modified from Fritsche and Hofrichter, 2000).

Although aerobic microbial hydrocarbon metabolism has been extensively investigated, the same is not true in what anaerobic hydrocarbon metabolism are concerned. Degradation of hydrocarbons by oxygen-respiring microorganisms has been known since the beginning of the 20th century; however, utilization of hydrocarbons under anoxic conditions has only been investigated during the late 1980s (Widdel and Rabus, 2001). Work with

microbial consortia in the field, in enrichment cultures, and in microcosms has illustrated that hydrocarbons such as toluene, alkylbenzenes, benzene, naphthalene, *n*-alkanes, branched alkanes, and hydrocarbon mixtures can be metabolized under anaerobic conditions (*e.g.* Foght, 2008; Heider et al., 1999; Jahn et al., 2005; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Principles of anaerobic hydrocarbon metabolism have been elucidated in a number of representative microorganisms. These reactions may take place under Fe(III)-reducing, denitrifying, and sulfate-reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria (Van Hamme et al., 2003).

According to Widdel et al. (2006) there is evidence for at least five principally different mechanisms for an anaerobic activation of hydrocarbons:

(i) The assumed activation of methane in a "reverse methanogenesis";
(ii) The radical catalyzed addition of alkanes and many alkylbenzenes to fumarate yielding substituted succinates (*e.g.* Figure 1.6);
(iii) The dehydrogenation of ethyl- and propylbenzene yielding aryl-substituted secondary alcohols;
(iv) The hypothesized addition of a carboxyl or methyl group to unsubstituted aromatic hydrocarbons;
(v) The hydration of double bonds (assumed) and triple bonds (demonstrated experimentally) in alkenes and acetylene, respectively.

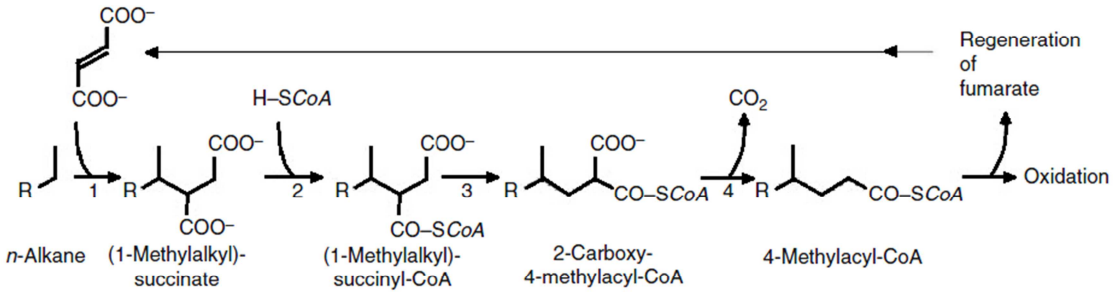

Figure 1.6 - An example of the proposed reactions for the anaerobic activation and further metabolism of alkanes hydrocarbon (Source and details in Widdel et al., 2006).

1.3.2 Bioremediation processes

Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. Bioremediation should be given preference in decision making for oil spill cleanup in coastal wetlands when the oil concentration is not high enough to destroy the ecosystem. The feasibility of bioremediation methods also depends on various factors (*e.g.* see section 1.3.3.1). Although bioremediation can also be used as *ex situ* treatment technique, such as landfarming, composting and biopiles, it involves the previous negative consequences of excavation or removal of contaminated sediments from ground and moving the contamination elsewhere. Therefore the main advantage of the *in situ* bioremediation processes is that it allows sediment to be treated without being excavated and transported, resulting in less disturbance of site activities, at a relative low cost. The most important biological processes that can take place in salt marsh are:

(i) **Natural attenuation**

Bioremediation which occurs without human intervention is called natural attenuation. This natural attenuation relies on natural conditions and behavior of indigenous soil/sediment microorganisms (Mills et al., 2003). When chosen as the only cleanup method, a monitoring program is still required to assess the performance of natural attenuation. This approach is viewed as the most cost-effective option for the cleanup of oil spills in coastal wetland environments. However, intrinsic bioremediation often takes a long time to complete because of population size of the indigenous degrading microorganisms are low and/or not stimulated (Zhu et al., 2004).

(ii) **Biostimulation**

Biostimulation uses microbial communities already present in sediments, and then improves the degradation potential of those bacteria capable of the desired degradation by adding nutrients or suitable electron donors/acceptors that are not available in suitable concentrations in the sediments (Nikolopoulou

and Kalogerakis, 2009). It has been shown that the degradation of PHC by a given native microbial population can be favored by the presence of the required nutrients in the contaminated site (Delille et al., 2004). This strategy promotes biodegradation, especially in environments where nutrients are often limiting (Burns et al., 1999).

(iii) Bioaugmentation

Bioaugmentation or seeding is the addition of highly concentrated microbial populations (single strains or consortia) capable of degrading the pollutant in the contaminated site (Gentry et al., 2004). The rationale for this approach includes the argument that indigenous microbial populations does not possess the metabolic routes necessary to metabolize complex mixtures, such as petroleum, and that seeding may reduce the lag period before bioremediation begins (Leahy and Colwell, 1990). The commonly options used for bioaugmentation are: addition of a pre-adapted pure bacterial strain or consortium; introduction of genetically engineered bacteria; and addition of biodegradation relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms (El Fantroussi and Agathos, 2005). Nevertheless, this approach causes apprehension, which limits its use. On one hand, added bacteria may not be able to compete with the indigenous and well-adapted population (Venosa et al., 1992; Zhu et al., 2004). On the other hand, the introduction of genetically engineered microorganisms faces greater difficulties in winning social acceptance, and the efficiency under laboratory conditions are not necessarily effective in situ (Sayler and Ripp, 2000). More recently, the addition of autochthonous pre-grown microbial cultures is proposed to overcome these difficulties (Hosokawa et al., 2009). The use of indigenous bacterial strains may be a valuable bioremediation strategy for cleaning the environment from hydrocarbon pollutants.

(iv) Oxygen amendment

Recent field studies on oil bioremediation have demonstrated that the availability of oxygen, not nutrients, is likely the limiting factor for oil

biodegradation in coastal wetlands (Natter et al., 2012). Salt marsh sediments are inundated with water due to a semidiurnal tidal cycle effect, and diffusion rates of oxygen through the sediment are very slow. Moreover, oxygen in the interstitial water is quickly depleted by aerobic metabolism of detritus that is abundant in wetlands. Many of the oxygen amendment technologies developed in terrestrial environments (*e.g.* tilling, bioventing, biosparging, and the addition of chemical oxidants), are currently not considered viable options for use in coastal wetlands (Zhu et al., 2004; Venosa et al., 2002). Therefore, further research is still required to explore cost-effective oxygen amendment techniques for the bioremediation of coastal wetlands.

(v) Phytoremediation

Phytoremediation is a plant-assisted bioremediation. It is an emerging cost-effective technology that shows potential for accumulating, immobilizing, and transforming persistent contaminants from soil, sediments, and water. It is a technology applicable to sites containing nutrient, organic or inorganic pollutants. In Table 1.2 are stated several phytoremediation applications. The six types of phytoremediation techniques are classified based on the contaminant type and fate: phytoextraction, phytotransformation, phytostabilization, rhizodegradation, rhizofiltration and phytovolatilization. For each of these mechanisms, a large variety of plant species have been assessed for their phytoremediation potential. There are numerous literature reviews which summarize information on plant species that play a role in the phytoremediation (*e.g.* Juwarkar et al., 2010).

The main advantages of phytoremediation in comparison with classical remediation approaches can be summarized as: i) can be applied in situ, and has potential versatility to treat a diverse range of hazardous materials; ii) less disruptive to the environment, since avoids excavation and heavy traffic; iii) economically competitive and has favorable public opinion.; iv) improves soil quality by preserving the natural structure and texture of the soil, and prevents erosion. Nevertheless, there are some limitations that it is necessary to consider carefully before it is selected for site remediation: i) long duration of time for remediation, ii) difficulty establishing and maintaining vegetation at some sites with high toxic levels.

Table 1.2 - Overview of phytoremediation applications (adapted from Vidali, 2001).

Technique	Plant mechanism	Contaminants	Substract
Phytoextraction	Uptake and concentration via direct uptake into the plant tissue	Metals	Soils
Phytotransformation	Plant uptake and degradation	Organic compounds	Surface water, groundwater
Phytostabilization	Root exudates precipitate and contaminants become less available	Metals	Soils, groundwater, mine tailing
Rhizoremediation	Enhances microbial degradation in rhizosphere	Organic compounds	Soils, groundwater within rhizosphere
Rhizofiltration	Uptake into plant roots	Metals	Surface water and water pumped
Phytovolatilization	Plants evaportranspirate	*e.g.* selenium, mercury, volatile hydrocarbons	Soils and groundwater

There has been increasing interest in the phytoremediation of PHC as widespread and recalcitrant pollutants (Schnoor et al., 1995). In terrestrial environments, Frick et al. (1999) indicated that microorganisms in the rhizosphere of plants are the primary mechanism for PHC degradation. Although limited studies have been carried out on the effectiveness of phytoremediation to enhance oil degradation in coastal wetland environments, rhizoremediation seems to be the best approach to remediate hydrocarbon contaminated salt marsh sediments, and therefore described in better detail in the following section.

1.3.3 Rhizoremediation: enhanced rhizosphere biodegradation

The rhizosphere has been defined as the volume of soil adjacent to and influenced by plant roots (Yateem et al., 2007). Rhizoremediation (also called rhizodegradation) involves the breakdown of contaminants in soil as a result of microbial activity that is enhanced within the rhizosphere (Kuiper et al., 2004). During rhizoremediation (Figure 1.7), plant root exudates (*e.g.* alcohols, organic acids, sugars, and other carbohydrates) can help stimulate the survival

and action of microorganisms in the soil. Plant-produced compounds may serve as co-substrates facilitating microbial degradation of the more recalcitrant compounds (Walker et al., 2003). This has been referred to as co-metabolism. Moreover, soil microbes digest organic pollutants producing harmless by-products. This form of fostering microbial activity, involving the interaction between plant and microorganisms, in terrestrial soils is well known, and has been referred in the literature as the "rhizosphere effect" (Olson et al., 2003).

The objective of rhizoremediation is to increase microbial numbers and activity, and the exploitation of that increased microbial activity to enhance biotreatment. Microbial densities in the rhizosphere are suggested to be 1 to 4 orders of magnitude higher than in bulk soil (Pilon-Smits, 2005). Although the importance of the rhizosphere community for degradation of pollutants in terrestrial soils has been recognized, very little is known about the survival, proliferation, activity, and exact composition of degrading populations in the rhizosphere of salt marsh plants.

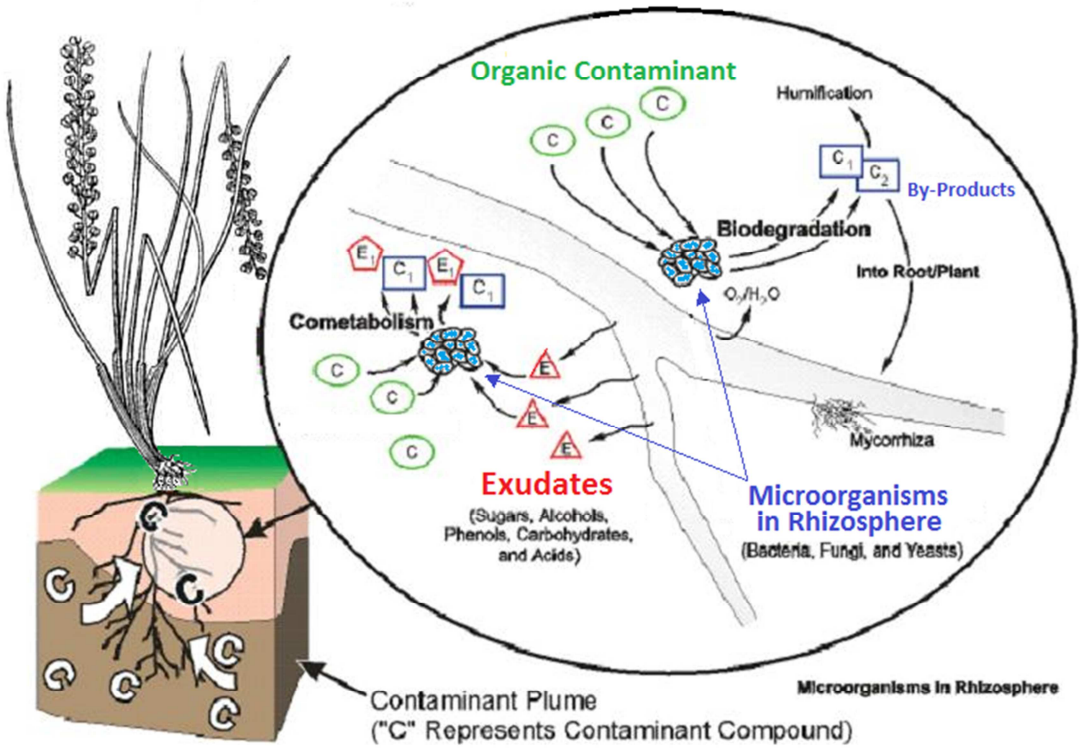

Figure 1.7 - Rhizoremediation: plant-microorganisms interactions in the biodegradation of organic pollutants in contaminated soil or sediment (modified from http://systemsbiology.usm.edu/BrachyWRKY/WRKY/Rhizodegradation.html).

Moreover, as stated earlier, oxygen is likely a limiting factor for oil biodegradation in marine wetlands, and no feasible technique is currently available for increasing the availability of oxygen in these environments. However, plants may be an appropriate technology for increasing the oxygen concentration. If we consider the plant as a solar-driven biological pump, on one hand, during transpiration water in the rhizosphere is attracted with its root system, accumulating water-soluble pollutants at the vicinity of roots (Erickson, 1997). On the other hand, internal photosynthetic oxygen produced is transferred from the shoot system to roots, and released into rhizosphere (Pezeshki and DeLaune, 2012), which may enhance aerobic microbial transformations of organic compounds at the vicinity of roots.

1.3.3.1 Factors affecting Rhizoremediation

In order to determine the outcome of any remedial strategy, an understanding of the interplay between the biotic and abiotic factors is important. There are several parameters that can influence PHC bioremediation in soils, and some of the parameters affect rhizoremediation processes directly while others impact by altering the bioavailability of the pollutant. These parameters are well characterized for terrestrial environments, even though with some differences mostly may be applied to salt marsh sediments, and can be divided in:

(i) **Environmental parameters**

The control and optimization of rhizoremediation processes is influenced by a complex system of several environmental factors. These factors include: texture and structure of soil, temperature, pH, OM content, moisture, oxygen availability, nutrients, salinity, solar radiation and weathering. These factors affect the properties and bioavailability of hydrocarbons, germination and productivity of plants, and colonization and growth of rhizosphere microorganisms. Table 1.3 outlines some optimum environmental conditions for the biodegradation of hydrocarbon contaminants in soil. The soil physical characteristics, texture and structure, respectively the mixture of different sizes and the arrangement of mineral particles, can limit the bioavailability of petroleum contaminants. Sand does not bind molecules as readily as silt or

clay, so the bioavailability of hydrocarbons is higher in sandy soils (e.g. Carmichael and Pfaender, 1997). Clay and OM content also affects microbial populations via their ability to form soil aggregates (Paul, 2007). Moreover, high OM contents can strongly adsorbed PHC into the soil system (Otten et al., 1997), decreasing PHC bioavailability to plants, although not necessarily to soil microorganisms (Leahy and Colwell, 1990).

Table 1.3 - Environmental conditions affecting rhizoremediation (adapted from Vidali, 2001)

Parameter	Condition required for microbial activity	Optimum value for hydrocarbon degradation
Soil moisture	25-28% water holding capacity	30-90%
Soil pH	5.5-8.8	6.5-8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10%	10-40%
Temperature (°C)	15-45	20-30
Nutrient content	N and P for microbial growth	C:N:P = 100:10:1
Type of soil		Low clay or silt content

Most bacteria operate optimally at a near neutral pH, while fungi tolerate more acidic conditions (Leahy and Colwell, 1990). Very low or high pHs inhibit bacterial and fungal activity, decreasing the rate at which hydrocarbons are degraded (Lewis et al., 1984). However, the main effect of pH can be via alteration of nutrient availability. Nitrogen, phosphorus, potassium, sulfur, calcium and magnesium availability decreases steadily as pH drops below 6 (Brady and Weil, 1996). When a major oil spill occurs in salt marshes, contamination with hydrocarbon affects the carbon:nitrogen (C:N) ratio and can lead to limited nutrient availability for oil degradation (Hutchinson et al., 2003). Low nutrients availability has been described as an inhibitory factor for plant growth (Cipollini and Bergelson, 2001), which in turn can negatively affects phytoremediation. Therefore addition of fertilizers can improve hydrocarbon degradation rates (Lin and Mendelssohn, 1998).

Temperature affects plant and microorganism growth, and can have profound effects on sediment matrix such as volume, redox potentials and physiochemical state of hydrocarbons. The rate of hydrocarbon degradation increases with increasing temperature (Wright et al., 1997).

Unlike terrestrial soils, the sediments of coastal wetlands are saturated or flooded with water due to tidal cycles. Flooding may be considered as a major physical disturbance that can result in large changes in soil and sediment biogeochemical characteristics such as redox potential, pH and OM decomposition (*e.g.* Hambrick et al., 1980; Nyman and DeLaune, 1991). Flooding is known to be associated with anaerobic conditions (Nyman and DeLaune, 1991), and as seen previously, the most effective HD are aerobic, therefore the deficiency of oxygen can negatively affect the biodegradation process (Shin et al., 2000).

(ii) Biological parameters

Biological factors that may affect rhizoremediation include hydrocarbon degradation ability of microorganisms associated to roots, plant root architecture and growth rate, and exudation.

Not all species will tolerate the presence of hydrocarbon contamination (Tesar et al., 2002), or be able to effectively enhance remediation of hydrocarbons from the soil (Madsen and Kristensen, 1997). Therefore, the selection of suitable plant species for rhizoremediation is an important consideration. First, plants should be native from the area for which they are being used. There can be ecological risks associated with introducing species to an area, and the use of native species protects local biodiversity, and they are adapted to the prevailing environmental conditions (Gaskin et al., 2008).

Research has shown that plants, like grasses, with highly branched and fibrous root systems are potential candidates for the rhizoremediation of hydrocarbons (Aprill and Sims, 1990; Merkl et al., 2005). This root system covers a larger volume of soil and could support greater rhizosphere-contaminant-microbe interactions (Aprill and Sims, 1990; Yateem et al., 2007). However, a lack of knowledge regarding the role of the root system in hydrocarbons rhizoremediation is still evident. In salt marsh sediments it

would be important to found plants with high oxygen diffusion by roots. Oxygen diffusion in roots is determined by anatomical, morphological, and physiological characteristics (Colmer, 2003). Plants with highly branched and fibrous root systems have a greater area for oxygen diffusion; and probably will have a higher stimulation on aerobic HD.

It is well known that composition of the microbial populations in the rhizosphere may vary with plant species, the composition of the root exudate, root type, plant age, environmental factors, and exposure to pollutants (Chaudhry et al., 2005; Kuiper et al., 2004). Nevertheless, research should focus on plants that specificaly increase HD population density. Numerous studies have attempted to elucidate the specific impact of exudates on hydrocarbon degradation potential. Root exudates have been shown to stimulate both non-specific (Da Silva et al., 2006; Miya and Firestone, 2001) and specific (Yoshitomi and Shann, 2001) increases in HD populations. Specific components released by plants, including organic acids, amino acids, and phenolic compounds, can stimulate or repress expression of genes involved in hydrocarbon degradation. Researchers have begun to elucidate the nature of these interactions, but their complexity ensures that it is no easy task.

Plants with particular morphological characteristics (*e.g.* type root systems), coupled with growth characteristics (*e.g.* tolerate hydrocarbon contamination) and physiology (*e.g.* root exudates that stimulate HD) seems to be the ideal for the *in situ* treatment of hydrocarbon contaminated soil.

1.4 The study area

About 6,000 ships cross the ocean every day carrying 4 million tons of oil cargo (Lorenzo et al., 2009). Atlantic coast of the Iberian Peninsula is one of the main routes of oil cargo (Figure 1.8-A); therefore, there is a potential hazard due to oil spill accidents (Solana-Ortega and Solana, 2007). In the last 40 years, six major oil spills (Figure 1.8-B) occurred in NW Iberian Peninsula, as a result of tanker accidents such as “Polycommander”, 1970; “Jakob Maersk”, 1975; “Urquiola”, 1976; “Andros Patria”, 1978; “Aegean Sea”, 1992; and “Prestige”, 2002.

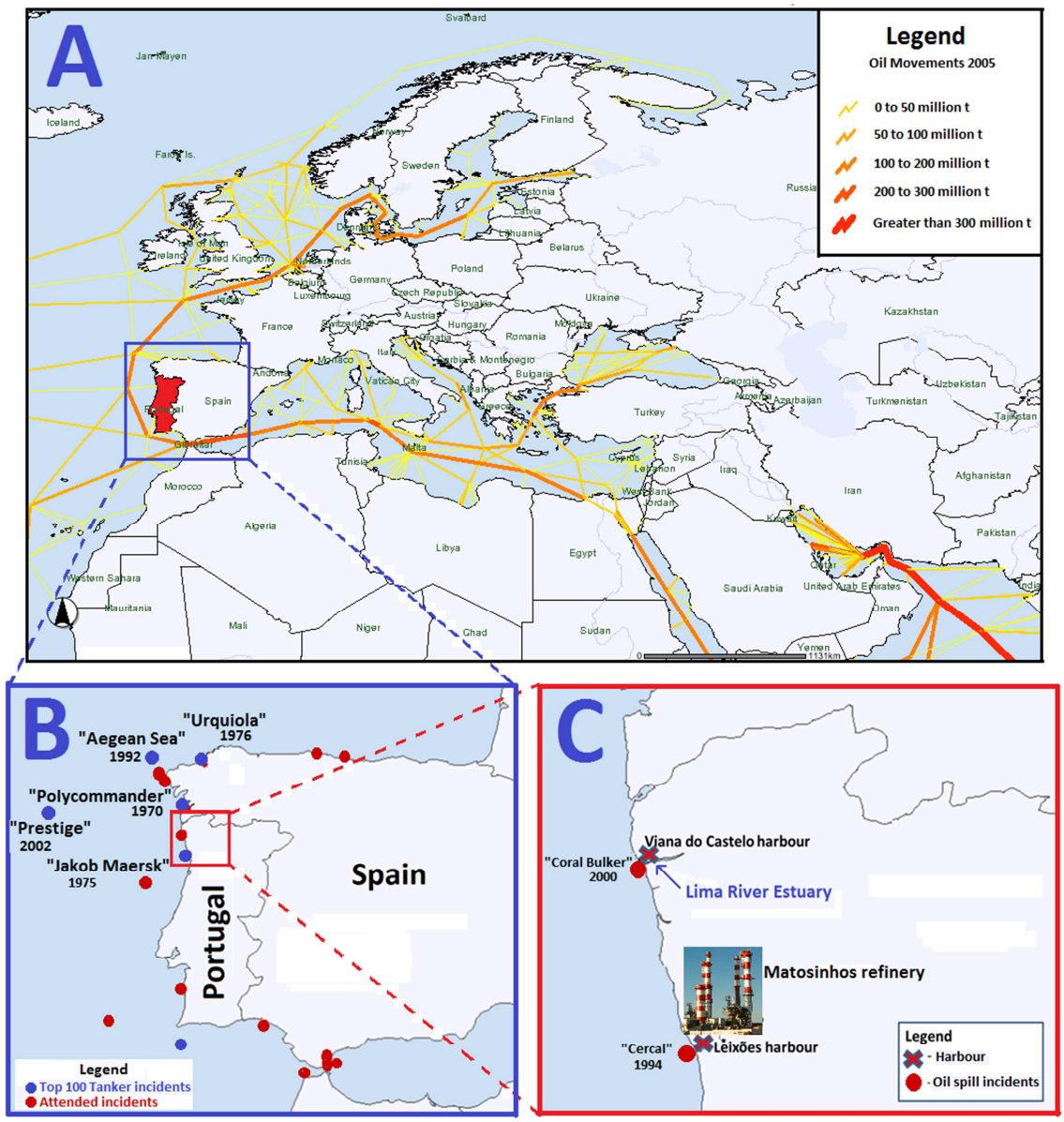

Figure 1.8 - **A)** Oil movements and amount transported in Europe during the year 2005. **B)** Major oil spills occurred in the coast of the Iberian Peninsula as a result of tanker accidents and attended incidents. **C)**. Location of the study area (Lima River Estuary), the two maritime harbors and refinery in NW of Portugal. Figures modified from http://www.itopf.com/website/ITOPFWebGIS/viewer.htm.

In a recent accident, the oil tanker "Prestige" sunk off the coast of Galicia, NW Iberian Peninsula, spilling 64,000 tons of oil. Considering the extension of the area affected by the black tides (more than 1,000 km), all estuaries on the coast of Galicia were affected by the oil spill (Andrade et al., 2004). Although the black tides did not reach the coast of Portugal, the southern continuation

of the Galician coast, it is possible that some of the released fuel oil and/or some of its components reached the NW of the Portuguese coastal zones (Tim-Tim et al., 2009).

The study area, Lima River Estuary (41.41ºN; 08.48ºW (WGS84)) is the end member of an international watershed located in NW of Portugal (Figure 1.8-C). The NW Atlantic coast of Portugal is also exposed to petrochemical contamination due to the presence of oil refining industry and two major seaports at Leixões and Viana do Castelo (Figure 1.8C). The Lima River mouth has its right bank modified by a city, Viana do Castelo (population 86 000), and has been subjected to several anthropogenic actions impacts (Araújo et al., 2010). There is an important harbor, leading to continuous petrochemical contamination through the activity of commercial and fishing vessels (Lima et al., 2007). Moreover, in 2000, the bulk carrier 'Coral Bulker' ran aground at the entrance of the estuary, spilling 630 t of heavy fuel oil and 70 t of diesel oil, and severely affected the area (Moreira et al., 2004).

The most industrialized areas are concentrated at the vicinity of the Lima estuary, in Viana do Castelo, and also further upstream near Ponte de Lima and Ponte da Barca, 23 Km and 40 Km respectively from the river mouth. Additionally, the Lima estuary suffers from several sources of disturbance, such as a paper mill in the upper estuary, as well as input from agricultural run off and urban and industrial sewage, which discharges nutrients and other substances that are transported into the estuarine area (Costa-Dias et al., 2010; Guimarães et al., 2009). Over several decades, the Lima estuary has been negatively affected by several heavy modification construction projects that were designed to serve commercial navigation and fisheries in this important Portuguese harbor. All of these interventions have modified the physical nature of the lower part of the estuary.

The Lima River is an international water body, with a source at about 950 m altitude in the Sierra S. Mamede, located in the province of Orense–Spain (ARH Norte, 2000). The Lima River basin is oriented lengthwise along a Northwest-Southwest direction and passes through granite outcrops with some schist and sand deposits (ARH Norte, 2000). The Lima River basin is located in one of the rainiest regions of Portugal. Consequently the Lima River valley is considered to be one of the richest regions in terms of hydrological resources, mainly

used for water supply, agricultural irrigation and hydropower generation. The Lima basin has an overall moderate to high ecological importance, which is recognized in terms of relevant ecosystems and important biodiversity (ARH Norte, 2000). There are several areas designated within the Natura 2000 network, Corine biotope and Protected Areas criteria, as well as areas classified accordingly to the Habitats Directive (92/43/EEC), Annex I (Costa-Dias et al., 2010; Rede Natura, 2000). In terms of vegetation, the Lima River exhibits an important biodiversity, mainly along the banks but also in terms of aquatic vegetation.

The Lima estuary

The Lima River has a small open estuary expanding East-West, where the river mouth was partially obstructed by a 2 km long jetty that deflects the river flow to the south (Ramos et al., 2006). This thesis study was undertaken within the initial 5 km stretch of the Lima River estuary. This area comprises a high variety of habitats, such as rocky substrata (essentially represented by sea walls in the lower part of the estuary), constructed and natural embayments, sandy-muddy intertidal banks, intertidal saltmarshes, sandy islands and small streams.

For this study, the Lima estuary was divided into three geomorphologically distinct zones: lower, middle and upper estuary (Figure 1.9). Sampling sites were chosen in the middle and lower estuary where salt marshes are found. These designations were based on morphology, bathymetry, salinity range and the presence/absence of saltmarsh (Ramos et al., 2009).

The lower estuary, located in the initial 2.5 km, is a narrow, deep navigational channel that is highly industrialized, with walled banks and includes a large shipyard, commercial seaport, and a fishing harbor. The river mouth is an artificial and deep channel with two protection piers. The average depth of 10 m is maintained by constant dredging activity. Salinity is generally higher than 25, and despite the depth, the water column exhibited slight vertical stratification (Ramos et al., 2010). The constant dredging of the navigational channel, promotes erosion of the banks and sand islands, and can also compromise the foundations of emerged structures, such as the bridge pillars

(Alves, 1996). Sedimentological variations along the lower estuary show that samples are in generally represented by small size particles (mainly muddy/slightly muddy sands) and contain higher levels of OM (Cardoso et al., 2008).

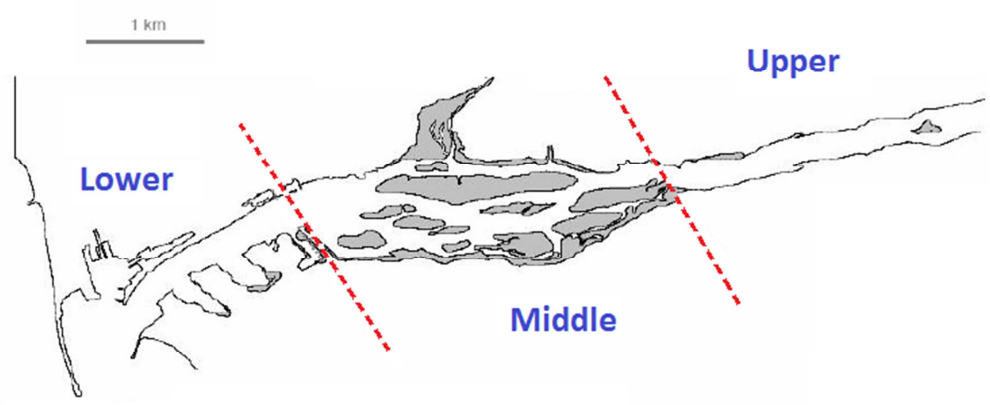

Figure 1.9 - Lima estuary division into three geomorphologically distinct zones: lower, middle and upper estuary. Shaded area represents salt marshes.

The middle estuary represents the largest part of the estuary. Sediments are characterized by a coarser fraction (muddy sands), and a lower fine fraction content, OM also decreases significantly compared to the lower estuary (Cardoso et al., 2008). This region encompasses the main course of the river and a large saltmarsh bank, mainly colonized by the common rush (*Juncus spp.*), in association with other plants (Ramos et al., 2010). According to Alves (2004) the *Triglochino striatae-Cotuletum coronopifoliae* association occurs on the north-western coast of Portugal due to humid to sub-humid mesomediterrenean bioclimate. This perennial sub-halophilic formation colonize humid depressions, predominated by *Juncus maritimus*, *Phragmites australis*, *Cotula coronopifolia*, and the presence of *Triglochin striata*, *Polypogon maritimus*, *Spergularia marina*, *Paspalum vaginatum*, *Agrostis pseudopungens*, *Lolium perenne*, *Aster tripolium s.l.*, *Spartina patens*, *Scirpus compactus*, and *Samolus valerandi* (Costa et al., 2009a; Honrado et al., 2002).

Further upstream, the estuary continues as a narrow and shallow channel (3 m), where depth decreases upstream. This sector still remains in an almost

natural state with undisturbed banks mostly constituted by sandy/slightly sandy muds (Cardoso et al., 2008).

1.5 Objectives and organization of the thesis

The main objective undertaken in this thesis is the contribution to the understanding of the specific plant-microbe interactions that facilitate PHC degradation in the rhizosphere of salt marsh plants.

The main objective was pursued through a series of specific objectives and studies designed in the following tasks:

(i) A sampling program during a phenological cycle of several salt marsh plants (representative from the River Lima Estuary) to characterize the rhizosphere, and un-colonized sediment at vicinity of plants, in terms of hydrocarbon contamination, microbial communities and natural physicochemical characteristics.

(ii) Microcosms experiments in laboratory conditions to assess and compare hydrocarbon degradation potential of: 1) microorganisms associated to roots of distinct plants in similar sediments; 2) microorganisms in rhizosediments, with distinct natural characteristics, colonized by the same plant; and 3) microorganisms in rhizosediments, and in un-colonized sediments at vicinity of plants.

(iii) Mesocosms experiments in greenhouse, with environmental conditions similar to those found in the estuary, to assess and compare hydrocarbon degradation potential. For this task, plants were selected based on the capacity to foster HD, and in the HD capacity to decrease PHC contents in sediments (obtained in previous tasks). Additionally, two treatments were carried out simultaneously: 1) addition of enrichment culture of indigenous HD; and 2) addition of nutrients.

This thesis is structured in seven chapters. Chapter 1 provides a general introduction on estuaries and salt marsh, PHC remediation processes, particularly the plant-microorganisms associations, and the factors effecting hydrocarbon rhizoremediation in salt marshes. Also describes the motivation and objectives of the research.

The following five chapters are adapted from original papers, four published and one submitted, relevant to the topic of this thesis. Chapter 2 presents preliminary results from the sampling program that included all sites and plants selected and introductory results from microcosms experiments. Chapter 3 briefly outlines the influence of natural rhizosediments characteristics on associated microorganisms and hydrocarbons degradation potential. Chapter 4 emphasizes the influence of distinct salt marsh plants on HD abundance during a phenological cycle. Chapter 5 shows results from the bacterial community response to PHC in sediments characterized in the previous chapter. Chapter 6 evaluates the potential of bioremediation strategies for remove PHC in mesocosms.

Finally, in Chapter 7, general conclusions on the research performed are summarized, as well the major outcomes of the work and directions for future research.

1.6 Environmental relevance of the study

Crude oil spills in the marine environment are one of the major pollution problems worldwide. Notably, conventional techniques used for remediation of hydrocarbon are inappropriate for salt marshes environment. In fact, they are ecologically sensitive areas and, when impacted by oil spills, can trap large quantities of oil and therefore, becomes a challenge in terms of clean-up.

Clearly, environmental restoration from oil spills focuses on the need for environmental friendly strategies. Microbial biodegradation is one of the principal processes for removal of non-volatile crude oil components from oil-contaminated marine sediments. The provision of a viable rhizoremediation technology would offer an economically feasible and environmentally sustainable option for the remediation of hydrocarbon contaminated salt

marshes. The development of this technology may have extensive application to problems associated with hydrocarbon contaminated sites. Therefore, gaining a better understanding of the factors influencing the biodegradation of spilled oil and soil physico-chemical and biological functions is an important step in the assessment of the environmental impact of oil spills and in developing and/or improving existing biodegradation-based remediation strategies.

Worldwide research on rhizoremediation of hydrocarbons contaminated salt marsh sediments is mainly addressed in the USA. Little information is available on rhizoremediation in European estuaries; therefore, the present study can provide a baseline to temperate salt marshes located in Europe. Moreover, regarding the complexity of all factors (waterlogged sediment, environmental factors, plants and its associated microbes, and hydrocarbons) it could be well claimed that biodegradation of PHC in salt marsh sediments is one of the most complicated biological processes that man tried to engineer.

Chapter 2

Hydrocarbon degradation potential of salt marsh plant-microorganisms associations

Published in Biodegradation, 2011
Volume 22: 729-739

Hydrocarbon degradation potential of salt marsh plant-microorganisms associations

2.1 Introduction

Estuaries are often considered sinks for contaminants, receiving important anthropogenic inputs from the upstream catchments and from metropolitan areas and industries located on or near those areas (Almeida et al., 2004). Estuaries are dynamic, complex and unique systems that present both seasonal and spatial variability (Chapman and Wang, 2001). Petroleum hydrocarbons are among the most common contaminants bound to estuarine sediments (Chapman and Wang, 2001), giving rise to significant environmental concern (Daane et al., 2001). The heterogeneity and variability of grain size estuarine sediments along with their OM content can influence the sequestration of hydrocarbons (Kukkonen and Landrum, 1996; Wang et al., 2001). In addition, the periodic inundation of the estuarine environment due to tides, with the subsequent percolation of salt water, can enhance the sorption of hydrophobic chemicals to the sediment particles caused by "salt effects" (Brunk et al., 1997).

Temperate salt marshes, including those at estuarine sites, have an important ecological role since they are among the most productive ecosystems on Earth (Boorman, 1999; Costanza et al., 1997). Simultaneously, these ecosystems are extremely sensitive to pollutants, including oil pollution (Andrade et al., 2004). In fact, some studies (*e.g.* Martins et al., 2008) highlighted the salt marshes capability to retain hydrocarbons in their sediments. As a result, it is important to clean and recover theses areas, which can be a difficult task (Zhu et al., 2004). Organic contaminants can undergo biodegradation as a result of the activity of sediment microorganisms giving less toxic, less mobile and/or less bioavailable products (Vidali, 2001). Accelerating the biodegradation of PHC in general is thus a major challenge in order to improve the performance and acceptance of cost-saving bioremediation techniques (Liste and Felgentreu, 2006).

In fact, the presence of vegetation can accelerate the bioremediation of sediments contaminated with PHC (Davis et al., 2002; Xu et al., 2006). In the specific case of soils, plants can alter the microbial community when introduced in a polluted area (Hartmann et al., 2009; Kirk et al., 2005), increasing the degradation of petroleum hydrocarbons relatively to that in bulk soil (Wang et al., 2008). It is well known that the rhizosphere is an ideal microhabitat for increasing the number of microorganisms (Hutchinson et al., 2003; Wang et al., 2008). The plant exerts a major influence on microbial communities through the release of a range of organic compounds, as root exudates, and eventually through nutrients released when the roots die and are degraded (Bais et al., 2006; Kuiper et al., 2004; Olson et al., 2003; Salt et al., 1998). Plants, on the other hand, benefit from the microbial turnover of root exudates and other soil organic and inorganic matter, which releases nutrients and enhances the soil structure (Olson et al., 2003; Prosser et al. 2006). The interactions between plant and microorganisms in the rhizosphere are complex and varied (Lambers et al., 2009; Prosser et al., 2006), being influenced by the plant species involved. Although hydrocarbon biodegradation in soils has been widely addressed, studies on salt marshes sediments are scarce, and it is still not clear how and to which extent the rhizosphere effect influences microbial communities and pollutants, namely petroleum hydrocarbon degradation (Daane et al., 2001; Merkl et al., 2006; Muratova et al., 2003) in theses estuarine ecosystems.

Therefore, the aim of this study was to give new insights on the influence of different salt marsh plant-microorganisms associations on petroleum hydrocarbons fate in a temperate estuarine environment, having in mind the need to increase the scientific knowledge for the development of alternative approaches to tackle coastal oil pollution, as the recent oil spill in the Gulf of Mexico highlighted.

2.2 Materials and Methods

2.2.1 The study area and sediment sampling

Sediment samples were collected in June of 2009 as well as in July - August of 2010 in the Lima River Estuary, the end member of an international watershed

located in NW Portugal. The urban-industrialized estuary has a large salt marsh area (Figure 2.1), and a mesotidal regime.

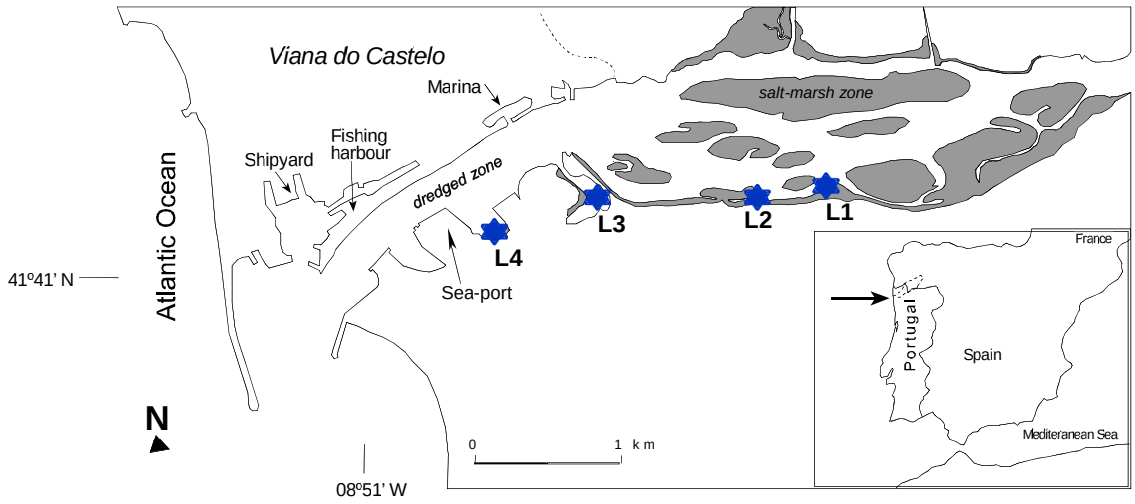

Figure 2.1 - Study area, Lima River estuary (North of Portugal), with the four sampling sites ( ): L1, L2, L3 and L4. Shaded area represents salt marshes.

During the 2009 survey, sub-surface sediments un-colonized and colonized (rhizosediments) by several plants (*Juncus maritimus*, *Phragmites australis*, *Triglochin striata* and *Spartina patens*) were collected into sterile plastic bags, at different sampling sites along the estuary (L1, L2, L3, L4, Figure 2.1). All sediments were collected between 5 and 15 cm, the depth with the higher plant belowground biomass in the case of colonized sediments. Samples were transported to the laboratory in the dark in refrigerated ice chests. At the laboratory ca. 40 g of each sediment sample was wrapped in aluminum foil and frozen at -20 ºC until total petroleum hydrocarbons (TPH) analysis. Remaining portions of the sediment were stored at 4 ºC for further treatment.

2.2.2 Sediment characterization

The determination of water and OM content (mean and respective standard deviation of three independent replicates) in the sediments was carried out according to the European Standard EN 13039:1999 methodology. OM content

was determined in dry sediments (at 100 ºC) by loss on ignition (4 h at 500 ºC).

To quantify particle size distribution, sediments samples were previously treated with a 30% hydrogen peroxide solution (Mikutta et al., 2005), and divided into five fractions in a mechanical shaker for sediment sieving. Although there are several different particle size limits that can be used (Nemes and Rawls, 2006), the adopted standard system was the followed: silt and clay (<0.063 mm), fine sand (0.063 - 0.25 mm), medium sand (0.25 - 1 mm), coarse sand (1 - 2 mm), and gravel (>2 mm). Each fraction was weighed and expressed as percentage of the total dry weight.

2.2.3 Microorganisms enumeration

Total cell counts (TCC) were obtained by 4',6'-diamidino-2-phenylindole (DAPI) direct count method (Porter and Feig, 1980; Kepner and Pratt, 1994). Triplicate sediment samples were immediately fixed with formaldehyde (0.2 µm-filtered), to reach a final concentration of 4% (v/v). Afterwards, to 0.1 g of homogenized samples were added 2.5 mL of saline solution (0.2 µm-filtered, 9 g L^{-1} sodium chloride) and 200 µL of Tween 80 solution (0.2 µm-filtered, 12.5% (v/v)), being fixed with 1 mL of formaldehyde solution (0.2 µm-filtered, 4% (v/v)). Samples were stirred at 150 rpm for 15 min followed by sonication for 20-30 s at low intensity (0.5 cycle, 20% amplitude). Sub-samples of fixed sediment samples were then stained with DAPI, and incubated in the dark for 12 minutes (Porter and Feig, 1980). Samples were filtered onto black Nuclepore polycarbonate filters (0.2 µm pore size, 25 mm diameter, Whatman) under gentle vacuum and washed with autoclaved 0.2 µm-filtered distilled water. Membranes were mounted in glass slides and cells counted at 1,875x on an epifluorescence microscope (Labphot, Nikon, Japan).

HD were estimated using a modified most probable number (MPN) protocol (Haines et al., 1996; Wrenn and Venosa, 1996), in 96-well microtiter plates. Pre-filtered (0.2 µm) of Arabian Light fuel oil (supplied by a local oil refinery) was the selective substrate for determination of total hydrocarbon degraders. Bushnell Haas medium (BH) supplemented with 2% sodium chloride was used as the growth medium for MPN procedures (180 µL BH/well). The fuel oil was

added to 5 x 12 wells (10 µL/well) after filling the wells with the growth medium. For each sample, 0.5 g of sediment was mixed in 1.5 mL BH and supernatant was diluted in a saline buffer solution containing 0.1% sodium pyrophosphate (pH 7.5) and 2% sodium chloride. Tenfold serial dilutions were performed, in the first row well of the microtiter plates, and the inoculation was made by adding 20 µL of each dilution to 5 wells. Five wells remained un-inoculated to serve as a sterile control. MPN plates were incubated for 2 weeks at room temperature. After incubation, 50 µL of filter sterilized Iodonitrotetrazolium violet (INT) (3 g L^{-1}) was added to each well. Positive wells were scored after overnight incubation at room temperature with INT.

2.2.4 Determination of total petroleum hydrocarbons concentration

Prior to TPH analysis, sediments samples were dried at room temperature until constant weight and sieved through a nylon net of 2 mm mesh in order to remove large particles and roots. For TPH measurements, a previous optimized method for soil samples was adapted for the sediment samples (Couto et al., 2012).

Briefly, about 1 g of sediment was mixed with anhydrous sodium sulphate (1:1 (w/w)) and tetrachloroethylene (≥99% spectrophotometric grade) (1:10 (w/v)) was added, being followed by an ultrasonic (Elma, Transsonic 460/H model) extraction for 30 minutes. The extracts were cleaned with deactivated silica gel (70-230 mesh), to remove non-mineral oil contaminants such as animal greases and vegetable oils and other polar compounds, and refrigerated until analysis, usually within 1 hour. The sample extracts were analyzed by Fourier transform infrared spectrophotometry (FTIR) (Jasco FT/IR-460 Plus) using a quartz cell of 40 mm path length (Infrasil I, Starna Scientific).

Calibration standards (in tetrachoroethylene) were prepared using a stock standard solution of equal volumes of isooctane (≥99% ACS spectrophotometric grade) and hexadecane (99%) solutions. TPH were quantified by direct comparison with the calibration curve. Quality control tests were conducted by analysing the certified reference material CRM350-100 (TPH in Sandy Loam Soil (C6 - C35), from Resource Technology Corporation). The results were within the prediction interval of expected TPH concentration.

Sample solutions spiked with known amount of hydrocarbons, yielded recoveries between 82 and 135 %. The mean and respective standard deviation of five independent replicates was calculated and the results were expressed on a dry weight basis.

2.2.5 Laboratory experiments

For the laboratory evaluation of the TPH biodegradation potential, experimental work involving degradation experiments was carried out with sediment samples, collected in 2010, in the L3 and L4 sampling sites (Figure 2.1). These experiments were restricted to those sites colonized simultaneously by the four plants mentioned above. The same procedures for sampling and handling described before were applied. However, the Arabian Light fuel oil was submitted to an aging process to simulate an oil spill by means of shaking the fuel oil overnight in BH medium. The experimental design adopted was, briefly, 10 ml (volume) of sediment samples were placed in 50 ml flasks, supplement with 20 ml BH medium and 0.5 ml of aged Arabian Light fuel oil. Initial triplicate sediment samples were collected for analysis of TPH, and considered as T_0 samples. The remaining flasks, with triplicate sediment samples, were incubated at room temperature in the dark in a mechanical stirring at 100 rpm. The flasks were also manually shaken once every day to improve blending between fuel oil and sediment. It has been previously suggested (Aichberger et al., 2005) that shaking flasks were the faster (2-4 weeks), cheaper and less sample requiring test method to predict biodegradation performance of hydrocarbons, with a good indication of hydrocarbon degradability. Simultaneously, sediment samples, not spiked with Arabian Light fuel oil, were incubated to verify the promoting effect of BH medium in the HD microorganisms. After 15 days of incubation, the sediment samples were removed and considered as T_{15} samples. All samples (including T_0) were frozen at -20 ºC (to stop microbial growth). After at least three days at -20 ºC, samples were left to dry at room temperature until constant weight. The TPH analysis in the dried T_0 and T_{15} sediments was performed as previously described. In the additional triplicate sediment samples, un-spiked with Arabian Light fuel, only HD MPN procedures were performed.

2.2.6 Statistical analyses

Microbial enumeration data were normalized by logarithm (log_{10}) transformation prior to statistical analysis. Significant differences ($P < 0.05$) between two means were evaluated using t-tests. Correlation factors ($P < 0.05$) were analyzed by correlation matrices. All statistical tests were performed using the commercial software Statistica (Version 9).

2.3 Results

2.3.1 Sediment characterization

Un-colonized sediments and rhizosediments collected around the different plants were characterized in terms of content in water and OM, and grain size distribution (Table 2.1).

Table 2.1 - Water (H_2O) and organic matter (OM) contents (mean and standard deviation, n = 3) and particle size fractions of dry un-colonized sediments (Sed.) and rhizosediments (rhizo) of *Juncus maritimus*, *Phragmites australis*, *Triglochin striata* and *Spartina patens*. Samples were collected at four different sampling sites (L1, L2, L3 and L4).

	Sample	% H_2O	% OM	size fraction percentage relatively to total weight				
				Silt + Clay	Fine sand	Medium sand	Coarse sand	Gravel
L1	Un-colonized Sed.	23 ± 2	2.3 ± 0.3	4.4	15	51	16	13
	J. maritimus rhizo.	23 ± 3	2.6 ± 0.2	6.0	18	42	18	17
L2	Un-colonized Sed.	37 ± 1	4.0 ± 0.2	1.4	51	38	5.2	4.4
	J. maritimus rhizo.	39 ± 4	3.74 ± 0.01	14	38	36	5.2	7.1
L3	Un-colonized Sed.	50 ± 1	5.4 ± 0.2	50	35	16	0.20	n.d
	J. maritimus rhizo.	53 ± 1	5.3 ± 0.1	26	57	17	n.d	n.d
	P. australis rhizo.	62 ± 1	6.00 ± 0.05	50	36	14	n.d	n.d
	T. striata rhizo.	53.2 ± 0.4	6.4 ± 0.2	41	53	6.0	n.d	n.d
L4	Un-colonized Sed.	27 ± 1	0.9 ± 0.1	1.6	50	46	1.5	1.1
	J. maritimus rhizo.	51 ± 2	5.85 ± 0.01	52	30	16	1.8	0.08
	S. patens rhizo	22 ± 2	2.0 ± 0.3	7.2	48	41	2.2	1.5

n.d: not detected

Sediments (both un-colonized sediment and rhizosediments) collect at L1, the uppermost sampling site, had coarser particles whereas sediments from L3 sampling site, in the lower estuary, had the smallest grain size (more than 80% of total particle size was inferior to 0.25 mm). Concomitantly, L3 was usually the site with the highest water and OM content.

When comparing rhizosediments with un-colonized sediments, a general tendency to register higher contents of water, OM and/or Silt and Clay fraction in rhizosediments was found. Nevertheless, significant ($P < 0.05$) differences could only be considered at sites L3 and L4. At L4, *S. patens* rhizosediment were more similar to the surrounding un-colonized sediment than to the *J. maritimus* rhizosediment. Nevertheless all sediments were significantly different ($P < 0.05$) in terms of OM content and fine-grained particles. At L3, *P. australis* and *T. striata* rhizosediments were significantly higher ($P < 0.05$) in OM content than the surrounding un-colonized sediment and the *J. maritimus* rhizosediment.

2.3.2 Microorganisms enumeration

The TCC and HD microorganisms abundance were estimated (Figure 2.2). The level of microbial abundance ranged from 10^7 to 10^9 TCC $g^{-1}_{wet\ sediment}$, whereas HD ranged from 10^4 to 10^8 MPN $g^{-1}_{wet\ sediment}$.

The results of total microbial abundance showed significant differences ($P < 0.05$) between un-colonized sediments and rhizosediments, with higher TCC in rhizosediments at all sites. Based on TCC, rhizosediments could be divided in two significantly ($P < 0.05$) different groups: A (L1 *J. maritimus* rhizo ≈ L2 *J. maritimus* rhizo ≈ L4 *S. patens* rhizo) and B (L4 *J. maritimus* rhizo ≈ L3 *J. maritimus* rhizo ≈ L3 *P. australis* rhizo ≈ L3 *T. striata* rhizo).

Considering HD abundance, significant differences ($P < 0.05$) between un-colonized sediments and rhizosediments were also found in all sites. In general, higher values were found in the rhizosediments, but the opposite occurred for *J. maritimus* rhizosediment in L3 sampling site. Also in L3 sampling site, HD abundance in *P. australis* rhizosediment was not significantly higher ($P > 0.05$) than at surrounding un-colonized sediment. Generally, no important differences ($P > 0.05$) were found between

rhizosediments of the different plants, although there were significant differences ($P < 0.05$) in the abundance of HD between the *J. maritimus* and *T. striata* rhizosediments collected at the same site (L3).

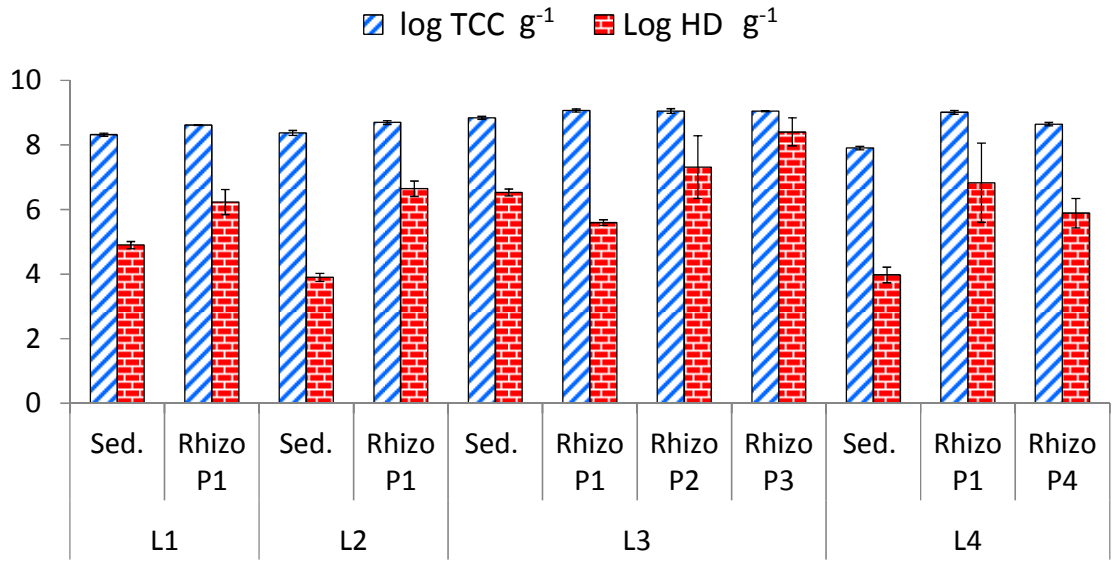

Figure 2.2 - Microbial abundance estimated by Total Cell Counts (log_{10} TCC g^{-1}, mean and standard deviation, n = 3) and Hydrocarbon Degraders microorganisms estimated by Most Probable Number (log_{10} HD g^{-1}, mean and standard deviation, n = 2) in un-colonized sediments (Sed.) and rhizosediments (Rhizo) of *Juncus maritimus* (P1), *Phragmites australis* (P2), *Triglochin striata* (P3) and *Spartina patens* (P4). Samples were collected at four different sampling sites (L1, L2, L3 and L4).

2.3.3 Total petroleum hydrocarbons concentration

The concentration profile of TPH in un-colonized sediments and rhizosediments are presented in Figure 2.3, and ranged from below detection level (0.032 mg g^{-1}) to 0.8 mg $g^{-1}_{dry\ sediment}$. The most notable aspect that emerged was a significantly higher ($P < 0.05$) TPH concentration in rhizosediments, except in L1 sampling site, the uppermost location. Also, the *J. maritimus* rhizosediment in L3 sampling site that was significantly ($P < 0.05$) lower than the surrounding un-colonized sediment.

Another perceptible feature was the difference between the L3 and the others sampling sites, that, with the exception of *J. maritimus*, had the highest statistically significant ($P < 0.05$) TPH concentrations.

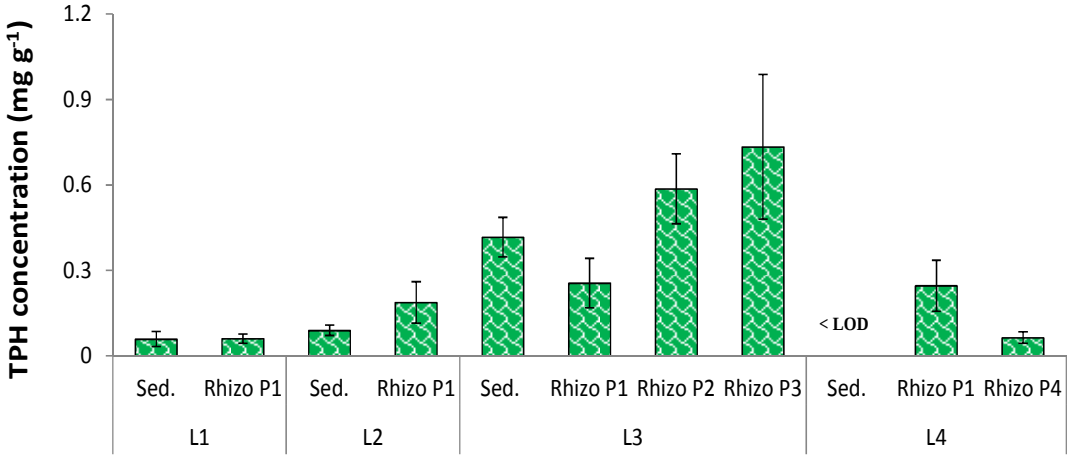

Figure 2.3 - Total petroleum hydrocarbons (TPH) concentrations (mg g^{-1}, mean and standard deviation, n = 5) in un-colonized sediments (Sed.) and rhizosediments (Rhizo) of *Juncus maritimus* (P1), *Phragmites australis* (P2), *Triglochin striata* (P3) and *Spartina patens* (P4). Samples were collected at 4 different sampling sites (L1, L2, L3 and L4). Limit of detection (LOD): 0.032 mg g^{-1}

The TPH concentrations in *J. maritimus* rhizosediment did not differed significantly ($P > 0.05$) among L2, L3 and L4 sampling sites, but were significantly ($P < 0.05$) lower at L1 sampling site. The TPH concentrations in *T. striata* (plant P3) and *P. australis* (plant P2) rhizosediments did not differed significantly ($P > 0.05$) between each other, being significantly ($P < 0.05$) higher than TPH concentrations in the other two plants (*J. maritimus* and *S. patens*) rhizosediments.

2.3.4 Laboratory experiments

The characteristics of the 2010 batch of sediments were similar to those retrieved in the previous year (data not shown), with the exception of HD abundance at site L3. In the latter case, no important differences ($P > 0.05$) were found between rhizosediments of the different plants, being the HD numbers of all three rhizosediments significantly ($P < 0.05$) higher than the surrounding un-colonized sediment.

Analysis of T_0 TPH (Table 2.2) showed differences between the several sediments, despite identical fuel oil addition. Nevertheless, a significant

positive correlation (r = 0.91, $P < 0.05$, n = 7) between these initial concentrations and those in un-spiked sediments was found, reflecting different capacities to retain hydrocarbons. Moreover, rhizosediments of all the studied plants presented higher rates of TPH degradation than un-colonized sediments. In addition, some differentiation in TPH degradation rates among different plants occurred. In the same vein, the stimulation effect of BH medium on HD abundance, after 15 days of incubation, varied among plants being the HD numbers from the *T. striata* rhizosediment the lowest. Finally, the HD abundance in medium supplemented with fuel oil could not be quantified because of methodological saturation

Table 2.2 – Total petroleum hydrocarbons (TPH) concentrations and hydrocarbon-degrading (HD) microorganisms enumeration (mean and standard deviation, n = 3) in laboratory experiments using un-colonized sediments and rhizosediments (Rhizo) of *Juncus maritimus*, *Phragmites australis*, *Triglochin striata* and *Spartina patens*. Samples were collected at two different sampling sites (L3 and L4). HD microorganisms were enumerated only in BH medium without fuel oil.

	L3				L4		
	Un-colonized sediment	*J. maritimus* Rhizo	*P. australis* Rhizo	*T. striata* Rhizo	Un-colonized sediment	*J. maritimus* Rhizo	*S. patens* Rhizo
TPH (mg g^{-1})							
T_0	26 ± 2	36 ± 3	45 ± 3	28 ± 3	1.9 ± 0.1	6.7 ± 0.6	1.7 ± 0.1
T_{15}	22 ± 4	25 ± 5	29 ± 10	22 ± 3	1.80 ± 0.07	5.7 ± 0.9	1.2 ± 0.2
Degradation	**15 %**	**30 %**	**35 %**	**23 %**	**3 %**	**15 %**	**28 %**
HD (log MPN)							
T_0	3.9 ± 0.2	6.2 ± 0.4	6.1 ± 0.4	6.7 ± 0.3	4.0 ± 0.3	5.4 ± 0.4	5.2 ± 0.2
T_{15}	8.1 ± 0.5	11 ± 3	9.49 ± 0.01	8 ± 3	7.5 ± 0.5	9.36 ± 0.09	8 ± 1
Promotion of HD	**111%**	**79%**	**56%**	**27%**	**86%**	**72%**	**63%**

2.3.5 Correlation factors

Considering both un-colonized sediments and rhizosediments, correlation factors between TPH concentrations and both biotic (HD microorganisms) and

abiotic (OM and Silt + Clay grain size fraction percentage) parameters were assessed and plotted in Figure 2.4. A significant positive correlation (r = 0.805, $P < 0.05$, n = 11) between HD and TPH concentrations were obtained, as well as between abiotic parameters and TPH concentrations (OM content (r = 0.836, $P < 0.05$, n = 11) and Silt + Clay grain size fraction (r = 0.810, $P < 0.05$, n = 11)).

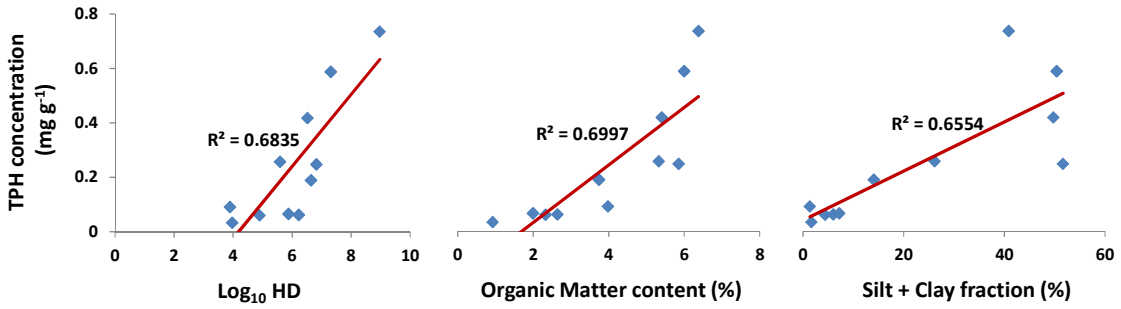

Figure 2.4 - Correlation plots of total petroleum hydrocarbons (TPH) concentrations vs hydrocarbon degraders microorganisms estimated by Most Probable Number (Log_{10} HD), TPH vs organic matter content and TPH vs Silt + Clay grain size fraction.

2.4 Discussion

The results obtained in this study suggest that salt marsh plants can positively influence the microbial community, by increasing total microbial abundance, and promoting the development of hydrocarbon degrading microbial populations on its rhizosphere. The results also pointed to a higher incorporation of TPH in sediments in contact with the plant belowground tissues in comparison to un-colonized sediments.

2.4.1 Relationships among TPH, OM and grain size

Discharges from municipal and industrial wastewater, urban runoff and oil leakage from boats and ships are possible sources of the TPH contamination in estuaries, including the Lima River estuary. Different sediments can have different capacities for collecting contaminants (Chapman and Wang, 2001),

and it has been demonstrated that sediment properties influence the distribution and concentration of hydrocarbons (Kim et al., 1999; Wang et al., 2001). The organic carbon fraction has been identified as the most important factor for the control of the concentrations of organic contaminants like hydrocarbons (Chapman and Wang, 2001). Several studies concerning PAH have demonstrated significant correlation between hydrocarbon concentration and OM content (Ke et al., 2005; Kim et al., 1999; Xu et al., 2007; Yang, 2000; Wang et al., 2001; Zhang et al., 2004). Our results have also shown a positive correlation ($P < 0.05$) between TPH concentrations and the OM contents. In fact, sediments (colonized and un-colonized) with higher OM contents were characterized by higher values of TPH (Table 2.1, Figure 2.3). A positive correlation ($P < 0.05$) between TPH concentrations and the Silt + Clay fraction was also observed, as expected as sediments with higher OM contents were in general those with an higher percentage of the Silt + Clay fraction.

The obtained results clearly showed that sediments containing high OM content might play an important role in adsorption, control, distribution and concentration of TPH, and in addition, grain size could also influence TPH distribution in sediments.

2.4.2 Plant influence on rhizosediment TPH retention

TPH concentrations were, in general, statistically higher in rhizosediments than in un-colonized sediments (Figure 2.3). Plants are a major source of organic matter into sediments since root exudates can polymerize with humic materials to form large and stable aggregate structures. These structures are conducive to sequestration of organic carbon, which increases OM content and, consequently, the binding of hydrocarbons (Gregory et al., 2005). We also observed a significant trend ($P < 0.05$) of higher OM content in rhizosediments, comparatively to the surrounding un-colonized sediments (Table 2.1). Moreover, the differences in the TPH concentrations among the different plants and the different sampling sites rhizosediments followed the same trend of the OM content (Table 2.1 and Figure 2.2). In fact, plants like *T. striata* and *P. australis* had not only higher OM but also higher TPH concentrations in their rhizosediments than the other two plants. Also, *J. maritimus* had similar TPH concentrations among the different sampling sites rhizosediments, except at

L1 where rhizosediment OM content was lower. The evidence that plants may be responsible for the movement of compounds into the rhizosphere, proposed by several authors (Liste and Alexander, 2000; Martins et al., 2008), may contribute to the importance of plants in the rhizosediments TPH retention. Consequently, the significant ($P < 0.05$) higher concentrations of TPH in rhizosediments, found in our study, may be explained by the movement of compounds towards the roots, due to the plant uptake of water and dissolved nutrients, from the surrounding sediments (Clothier and Green, 1997), followed by the subsequent sequestration onto OM content, as discussed above. Additionally, the exudation by plant roots of compounds like organic acids, aromatic acids and phospholipidic surfactants (Liste and Felgentreu, 2006 and references therein) may also facilitate the mobility of hydrophobic contaminants from the bulk sediment to the rhizosphere. Therefore, our results demonstrated that plants can effectively influence the distribution of TPH retaining, in general, more TPH around their belowground tissues than un-colonized sediments.

2.4.3 Hydrocarbons biodegradation potential by plant-microorganisms association

The present study demonstrated a significantly ($P < 0.05$) higher total microbial abundance in rhizosediments comparatively to un-colonized sediments, presumably because plants roots release oxygen and nutrients (especially small molecules such as amino acids, sugars and organic acids), creating an aerobic, nutrient-rich environment in which microbial activity was stimulated (Bais et al., 2006; Kuiper et al., 2004; Olson et al., 2003; Salt et al., 1998). This assumption is corroborated by the observed rhizosphere effect (Olson et al., 2003). Generally, in our study the total amount of microorganisms increased an order of magnitude in the rhizosediment, at the vicinity of plants roots, relatively to the surrounding un-colonized sediments. These findings are consistent with results reported in recent studies (Ho and Banks, 2006; Muratova et al., 2003; Nichols et al., 1997) but performed in soil rhizosphere.

Interestingly was the division of TCC data for rhizosediments into two significantly ($P < 0.05$) different groups: group A—L1 *J. maritimus* rhizo & L2 *J.*

maritimus rhizo & L4 *S. patens* rhizo) and group B—L4 *J. maritimus* rhizo & L3 *J. maritimus* rhizo & L3 *P. australis* rhizo & L4 *T. striata* rhizo. The TCC values in the group B were significantly ($P < 0.05$) higher than those of group A, a difference probably related to the OM content. In fact, we found a significantly ($P < 0.05$) higher OM content in the B group, and we also found a strong positive correlation ($r = 0.951$, $P < 0.05$, $n = 7$) between all TCC in rhizosediments and the OM contents. These results demonstrated the likely influence of OM, as an available carbon source on the growth and metabolic activity of rhizosphere microorganisms, which could be potentiated by plant roots.

Several studies have demonstrated the importance of the rhizosphere effect on the degradation of hydrocarbons, being most of these studies focused on terrestrial plants (see below). Recently, Wang et al., (2008) concluded that petroleum pollutants and plant rhizosphere promoted the increase of microorganisms that could degrade soil petroleum hydrocarbons. Corgié et al. (2003) also observed a bacterial gradient with higher numbers of hydrocarbon degrading bacteria in the soil closest to plant roots. In marine ecosystems, a positive correlation between the number of hydrocarbon-degrading microorganisms and oil pollution was found (Braddock et al., 1995 and references therein). Moreover, Leahy and Colwell (1990) have suggested that the levels of hydrocarbon degrader microorganisms generally reflect the degree of contamination of the ecosystem. Information regarding the influence that the rhizosphere of salt marsh plants might have on hydrocarbon degrading microorganisms is scarce.

Nevertheless, salt marshes rhizosphere are interesting sites to investigate the degradation of hydrocarbons because several factors favors their retention (Martins et al., 2008), and it contains a diverse population of hydrocarbon degrading bacteria (Daane et al., 2001). The present study revealed a significantly ($P < 0.05$) higher HD abundance in the rhizosediments which, attending to the earlier mentioned studies on soil rhizosphere, suggests the potential for higher degradation of hydrocarbons in this environment, compared to un-colonized sediments. In fact, we found a significant positive correlation ($P < 0.05$) between HD and TPH concentrations, demonstrating the salt marsh plant capabilities to retain hydrocarbons around their roots, and for fostering HD in its rhizosphere. Differences in degradation potentials were

confirmed by the laboratory experiments, which showed higher TPH degradation rates in rhizosediments than in un-colonized sediment (Table 2.2).

Not all plant species have the same potential for enhancing rhizoremediation, and climate, soil/sediment characteristics and salinity can also influence the success of the remediation process by one particular plant (Hutchinson et al., 2003). Although in our study *J. maritimus* rhizosediments had different sediment characteristics along the four sampling sites, the hydrocarbon content and HD enumeration were, in general, not significantly different ($P > 0.05$) among the four sampling sites.

On the other hand, plants can alter the microbial population, and these changes can be plant-specific (Kirk et al., 2005). These is a possible explanation for the differences in the numbers of hydrocarbon degraders among the rhizosediments of the different plants collected at the same site (*J. maritimus* < *P. australis* < *T. striata*), with statistically significant differences ($P < 0.05$) between *J. maritimus* and *T. striata*. This study suggests that the interaction between microorganisms and *P. australis* and *T. striata* probably have higher hydrocarbon degradation potential than the interaction with *J. maritimus* and *S. patens*. Differences in degradation potentials among plants were confirmed by the laboratory experiments, which showed different degradation rates among the different rhizosediments (Table 2.2). However, slightly lower degradation rates were observed for *T. striata* comparatively to the remaining plants. This fact can reflect an experimental limitation as, after 15 days incubation, a lower stimulation of HD microorganisms by the BH medium was observed for this plant.

More insights on plant–microorganisms interactions with regard to degradation process are needed, in particular the influence among salt marsh plant species, in order to fully ascertain the interactions of rhizosphere on hydrocarbon biodegradation. In fact, little information is available on salt marsh rhizoremediation; therefore, this report is one of the first attempts to describe the effect that salt marsh plants might have on hydrocarbon degradation in a temperate estuarine environment.

2.5 Conclusion

The results obtained in this study suggest that salt marsh plants can influence microbial communities by fostering the development of hydrocarbon-degrading microbial population in its rhizosphere, an effect observed for all plants species selected. This effect, combined with the plant capability to retain hydrocarbons around the roots, points out that salt marsh plant-microorganisms associations may actively contribute to hydrocarbon removal and degradation in temperate estuarine environment.

Chapter 3

Influence of natural rhizosediments characteristics on hydrocarbons degradation potential of microorganisms associated to Juncus maritimus roots

Published in International Biodeterioration and Biodegradation
http://dx.doi.org/10.1016/j.ibiod.2012.05.039

Influence of natural rhizosediments characteristics on hydrocarbons degradation potential of microorganisms associated to *Juncus maritimus* roots

3.1 Introduction

In the last decades, levels of petrochemical products in the environment, particularly in estuaries and coastal areas have increased (Lima et al., 2007). Marine oil spills, particularly large scale accidents, as the recent one in the Gulf of Mexico, have drawn great attention due to their catastrophic impact on the coastal environment. However, minor oil spills on coastal zones, as a result of municipal and industrial wastewater discharges, urban runoff, and oil leakage from boats and ships, are no less threatening to the environment and to public health, although they receive much less attention. Oil contamination affects all seashores, estuaries and rivers, causing the loss of biodiversity, destruction of breeding habitats of aquatic organisms and hazard to organisms, including man (Zhu et al., 2004).

Coastal intertidal zones, like salt marshes, are sensitive ecosystems that serve many ecological functions (Boorman, 1999; Lefeuvre et al., 2003), being often affected by oil spills and extremely vulnerable to hydrocarbons (Andrade et al., 2004). It is important to clean and recover these areas, which can be a difficult task (Zhu et al., 2004). Bioremediation can be considered a less damaging and cost effective cleanup approach, when compared to alternatives such as soil washing, incineration or disposal to landfill, often more damaging than the oil itself (Lin and Mendelssohn, 1998). Bioremediation of hydrocarbons often includes the biostimulation, where the growth of indigenous oil degraders is stimulated by the addition of nutrients, giving less toxic, less mobile and/or less bioavailable products (Vidali, 2001). The use of plants and their associated microorganisms is another approach for the treatment of contaminated areas, referred to as rhizodegradation or rhizoremediation (Gaskin and Benthan, 2010; Olson et al., 2003). In fact, the presence of vegetation can accelerate the

bioremediation of soils/sediments contaminated with petroleum hydrocarbons (Davis et al., 2002; Lin and Mendelssohn, 1998; Xu et al., 2006).

Petroleum hydrocarbons are among the most common contaminants bound to estuarine sediments (Chapman and Wang, 2001). However, sediment heterogeneity, the presence or absence of vegetation, variability of grain size, organic matter content along with the salt water intrusion can influence hydrocarbons sequestration (Brunk et al., 1997; Kukkonen and Landrum, 1996; Wang et al., 2001), and bioavailability (Amellal et al., 2001; Talley et al., 2002), as well as microbial communities responsible for their degradation (Marschner et al., 2004; Sessitsch et al., 2001). Therefore, hydrocarbon rhizoremediation processes should have into account sediment characteristics. Nevertheless, studies on this subject are scarce, particularly in estuarine areas, where a great variability of these characteristics can be found.

In this vein, the present study aimed to investigate the influence of natural characteristics of vegetated sediments on the associated microbial communities and on their hydrocarbon degradation potential. For that, microbial communities, HD microorganisms included, and petroleum hydrocarbon distribution and degradation rates were accessed on sediments colonized by *Juncus maritimus* (sea rush), a salt marsh plant. Sediments were collected at four different locations through the plant phenological cycle. To our knowledge, this is the first report on the influence of sediment characteristics on hydrocarbon degradation potential in salt marshes.

3.2 Materials and Methods

3.2.1 The study area

Atlantic coast of the Iberian Peninsula is one of the main routes of oil cargo; therefore, there is a potential hazard due to oil spill accidents (Solana-Ortega and Solana, 2007). In the last 40 years, six major oil spills occurred in NW Iberian Peninsula, as a result of tanker accidents such as Polycommander (1970); Jakob Maersk (1975); Urquiola (1976); Andros Patria (1978); Aegean Sea (1992); and Prestige (2002). The NW Atlantic coast of Portugal is also

exposed to petrochemical contamination due to the presence of oil refining industry and two major seaports (Leixões and Viana do Castelo).

The Lima River Estuary is the end member of an international watershed located in NW of Portugal (Figure 2.1). In 2000, the bulk carrier 'Coral Bulker' ran aground at the river mouth, spilling 630 t of heavy fuel oil and 70 t of diesel oil, and severely affected the area (Moreira et al., 2004). Also, there is an important harbor, leading to continuous petrochemical contamination through the activity of commercial and fishing vessels (Lima et al., 2007). The Lima Estuary is also a receiver of diffuse pollution originated from agriculture and from domestic and industrial waste discharges, including a paper mill (Costa-Dias et al., 2010; Guimarães et al., 2009).

The estuary has a semidiurnal and mesotidal regime, being vertically stratified only during the freshet period of the year (winter). During spring tides, salt intrusion can extend up to 20 km upstream (Ramos et al., 2006). A large salt marsh area spreads over the middle estuary, colonized by different plants, where *J. maritimus* (sea rush) is commonly found (Rede Natura, 2000; Costa et al., 2009a). The sampling sites (L1, L2, L3, L4; Figure 2.1) within the salt marsh area had different sediment characteristics and were colonized by individual assemblages of *J. maritimus*. These perennial plants, belonging to *Juncaceae* family, are an example of salt marsh native and ubiquitous specie of Portuguese salt marshes (Costa et al., 2009b). *J. maritimus* presented adventitious roots borne on the horizontal rhizome. The water/substrate temperature never goes below the freezing point in this estuary. Therefore, *J. maritimus* plants do not experience a true dormancy period, and do not present a live/death cycle with belowground (roots and rhizomes) and aboveground structures being quite stable during the phenological cycle (Almeida et al., 2006). In fact, individual plants died but simultaneously, in the same site, a new batch of plants grow up. Flowering occurs mainly in June-September (Sampaio, 1988).

3.2.2 Sediment sampling and characterization

Sediment samples were collected, at low tide, in the beginning of March, June, September and December of 2009 as well as in summer (July-August) 2010. In

each sampling site, approximately 1 kg of sediments colonized by *J. maritimus* (sediment in contact with the plant roots forward named rhizosediment) were collected into sterile plastic bags. In March, L4 rhizosediments were not sampled due to methodological/logistical problems. All rhizosediments were collected between 5 and 15 cm, the depth with the higher plant belowground biomass. Samples were transported to the laboratory in the dark, in refrigerated ice chests. At the laboratory, samples for DNA extraction were immediately frozen at -20 C. A portion of each rhizosediment samples was wrapped in aluminum foil and also frozen until TPH analysis. Remaining portions of the rhizosediment samples were stored at 4 ºC for other procedures.

The determination of water and OM content (mean and the standard deviation of three independent replicates) in the sediments was carried out according to the European Committee for Standardization (1999) methodology, by drying (at 100 ºC), followed by loss on ignition (4 h at 500 ºC). To quantify particle size distribution, sediment samples were previously treated with a 30% hydrogen peroxide solution (Mikutta et al., 2005), and divided into five fractions in a mechanical shaker for sediment sieving. Although there are several different particle size limits that can be used (Nemes and Rawls, 2006), the adopted standard system was the followed: Silt and Slay (<0.063 mm), fine sand (0.063-0.25 mm), medium sand (0.25-1 mm), coarse sand (1-2 mm), and gravel (>2 mm). Each fraction was weighed and expressed as percentage of the total dry weight.

The determination of electrical conductivity (EC) was made using a 1:5 sediment:water suspension according to Rayment and Lyons (2011). Sediment/water suspensions were prepared in triplicate. EC was measured using a calibrated YSI (Model 30) conductivity meter (YSI incorporated, Yellow Springs, OH, USA).

The pH value of the sediment was determined using a ratio of 1:5 sediment:CaCl2 solution (0.01 M) suspension according to Wilke (2005) using a calibrated Hanna (HI110) pH meter (Hanna Instruments Ltd, United Kingdom).

3.2.3 Determination of total petroleum hydrocarbons concentration

Prior to TPH analysis, sediment samples were dried at room temperature until constant weight and sieved through a nylon net of 2 mm mesh to remove large particles and roots. For TPH measurements, a previously optimized method was used (Couto et al., 2012). Hydrocarbons were extracted with tetrachloroethylene (99% Spectrophotometric grade, Sigma Aldrich) in an ultrasonic bath (Elma, Transsonic 460/H model) at room temperature for 30 min. The sample extracts were analyzed by Fourier transform infrared spectrophotometry using a quartz cell of 40 mm path length. Quality control tests were conducted by analyzing the certified reference material CRM350-100, with results within the prediction interval of expected TPH concentration.

Sample solutions spiked with known amount of hydrocarbons, yielded recoveries between 82 and 135%. The mean and the standard deviation of three independent replicates were calculated and the TPH results were expressed on a dry weight basis.

3.2.4 Microorganisms abundance

3.2.4.1 Total cell counts

TCC were obtained by DAPI direct count method (Porter and Feig, 1980; Kepner and Pratt, 1994). More details can be found in Ribeiro et al. (2011).

3.2.4.2 Hydrocarbon degrading microorganisms counts

HD microorganisms were estimated using a modified most probable number protocol (Haines et al., 1996; Wrenn and Venosa, 1996) in 96-well microtiter plates. Pre-filtered (0.2 mm) Arabian Light crude oil (supplied by an oil refinery) was the selective substrate for determination of total hydrocarbon degraders. Bushnell Haas medium supplemented with 2% sodium chloride was used as the growth medium. Further details can be found in Ribeiro et al. (2011).

3.2.5 Microbial community structure using automated rRNA intergenic spacer analysis (ARISA)

Microbial community structure was assessed in rhizosediment samples collected in September. Total DNA was extracted from wet homogenized sediment samples (three replicates) using a modification of the CTAB (bromide-polyvinylpyrrolidone-b mercaptoethanol) extraction protocol (Dempster et al., 1999) described by Barrett et al. (2006). Quality of extracted DNA was evaluated by visualization on 1.5% agarose gels and each DNA preparation was quantified with the Qubit fluorometer (Invitrogen). For ARISA, extracted DNA was amplified using ITSF (50-GTCGTAACAAGGTAGCCGTA-30) and ITSReub (50-GCCAAGGCATCCACC-30) primers set (Cardinale et al., 2004), which amplifies the ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson and Furhman, 2004). ITSReub was labeled with the phosphoramidite dye 6-FAM (6-carboxyfluorescein). PCRs were performed in duplicate 25 ml volumes containing between 2 and 6 ng of DNA, 400 nM of both primers, 200 mM dNTPs, 3Taq PCR buffer, 2.5 U Taq DNA polymerase, 2.5 mM MgCl and 1 mg bovine serum albumin. The PCR mixture was held at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 45 s, 55 ºC for 30 s, 72 ºC for 2 min, and a final extension at 72 ºC for 7 min. Duplicate PCR products were combined, examined on 1.5% agarose gel, purified using a GFX PCR DNA purification kit (GE-Healthcare) and eluted in 30 ml of water. The purified product was quantified using the Quant-it dsDNA assay kit, and the Qubit fluorometer (Invitrogen). A standardized amount of the purified PCR product was diluted (5 times) and mixed with ROX-labeled genotyping internal size standard (ROX 1000, Applied Biosystems). The sample fragments were run on the ABI3730 XL genetic analyzer undertaken by STABVIDA Sequencing Facilities (Lisbon, Portugal).

3.2.6 TPH degradation laboratory experiments

To investigate the influence of sediments characteristics on the TPH degradation potential, batch slurry tests, with rhizosediment samples collected in 2010 were performed. The same procedures for sampling and handling described before were applied. It has been previously suggested (Aichberger et al., 2005) that shaking flasks were the faster (2-4 weeks), cheaper and less

sample requiring test method to predict biodegradation performance of hydrocarbons, with a good indication of hydrocarbon degradability. For the experiments, sediment samples were placed in 50 ml flasks, mixed with BH medium, without (control) and with petroleum amendment (sediment:BH:petroleum of 20:40:1 (v/v)). The BH medium is a mineral-salts enrichment medium suitable for the promotion of HD, although it has a low content of nitrogen. Nitrogen is, generally, identified as the primary limiting nutrient for marsh vegetation (Crain, 2007). Therefore, to compensate a possible N-limitation, extra nitrogen was added (20 mM of NO_3 as 1.33 ml of 3 M KNO_3 solution p.a., Merck) to additional L1 and L3 rhizosediment samples with petroleum amendment. The Arabian Light crude oil was submitted to an aging process, to simulate an oil spill, by means of shaking the oil overnight in BH medium prior to addition to the flasks. Initial triplicates of each sediment sample with petroleum amendment were collected for analysis of TPH, and considered as T_0 samples. The remaining flasks, in triplicate for each sediment sample, were incubated at room temperature in the dark in a mechanical shaker at 100 rpm. The flasks were also manually shaken once every day to improve blending between oil and sediment. After 15 days of incubation, the sediment samples were removed and considered as T_{15} samples. TCC and HD MPN procedures were performed in all sediment samples as previously described. For TPH analysis, all samples (including T_0) were frozen at -20 ºC (to prevent microbial activity). After at least three days at -20 C, samples were left to dry at room temperature until constant weight. The TPH analysis in the dried T_0 and T_{15} sediments was performed as previously described.

3.2.7 Statistical and data analysis

The mean and standard deviation values of three replicates were calculated. Microbial enumeration data were normalized by logarithm (log_{10}) transformation prior to statistical analysis. Differences on HD, TCC, TPH and OM among rhizosediments were analyzed by parametric one-way ANOVA (analysis of variance). Whenever significant differences were detected, a multiple Tukey comparison test was performed. Correlation factors ($P < 0.05$) were analyzed by correlation matrices. All statistical tests were performed using the commercial software STATISTICA (data analysis software system), version 7, StatSoft, Inc. (2004).

Principal components analysis (PCA) was performed in the Primer 6 software package (version 6.1.11) (Clarke and Gorley, 2006) to ordinate the rhizosediment samples based on the values of environmental abiotic data. Measures included water and OM content, pH, EC and grain size distribution. Data were log (x + 1) transformed and normalized prior to analysis to have comparable (dimensionless) scales. A lower triangular resemblance matrix was created using Euclidean distances and then examined using a hierarchical cluster analysis. Dendograms were generated using the group average method. The Simprof test was used to test differences between clusters generated.

ARISA fragment lengths (AFL; corresponding to distinct peaks in the electropherogram) were analyzed by Peak Scanner Software (Applied Biosystems) and the data was transferred to a spreadsheet for further processing. Fragments that differed by less or equal to 2 bp were considered identical and fragments with Fluorescence Units below 200 were considered "background noise". Fragments less than 200 bp were removed since were considered to be too short internal transcribed spacer for bacteria. The resulting matrix of AFL and respective fluorescences for each sample were imported into the Primer 6 software package (version 6.1.11) (Clarke and Gorley, 2006). Data were normalized using the presence/absence pretreatment function and samples were then analyzed using the Bray-Curtis similarity method and a multidimensional scaling (MDS) plot was generated using the default parameters with a minimum stress of 0.01 to generate a configuration plot based on percent similarity.

3.3 Results

3.3.1 Sediment characterization and total petroleum hydrocarbons concentrations

3.3.1.1 Sediment characterization

Rhizosediments collected around the belowground tissues of *J. maritimus* were characterized in terms of water and OM content, pH, EC and grain size distribution (Table 3.1). Water and OM contents and Silt + Clay percentages were, generally, significantly ($P < 0.05$) different among rhizosediments,

increasing downstream until L3 site and stabilizing at the L4 site. EC and pH showed the same trend, but the differences were not significant ($P > 0.05$), being L3 rhizosediments the higher site for EC. The EC and pH of the estuarine water collected and analyzed "*in situ*" showed the same trend as EC and pH from rhizosediments (data not shown).

Table 3.1 - Water (H_2O) and organic matter (OM) contents, pH, electrical conductivity (EC, mS cm^{-1}) (mean and standard deviation, n = 3) and particle size fractions of dry rhizosediments of *Juncus maritimus* (Sea Rush). Samples were collected at 4 different sampling sites (L1, L2, L3 and L4) in four different seasons (in the beginning of March, June, September and December). In March, L4 rhizosediments were not sampled due to methodological/logistical problems.

Sample		% H_2O	% OM	pH	EC	particle size fraction percentage relatively to total dry weight				
						Silt + Clay	Fine sand	Medium sand	Coarse sand	Gravel
March 2009	L1	28 ± 1	3.4 ± 0.3	5.84 ± 0.05	2.53 ± 0.02	9.7	16.5	45.1	17.7	10.9
	L2	40 ± 3	4.65 ± 0.02	5.7 ± 0.1	4.20 ± 0.02	28.7	29.2	34.4	2.4	5.3
	L3	50 ± 1	5.5 ± 0.1	6.25 ± 0.06	8.3 ± 0.6	49.3	40.3	10.3	0.1	n.d
June 2009	L1	23 ± 3	2.6 ± 0.2	5.51 ± 0.04	2.58 ± 0.02	6.0	17.8	41.8	17.8	16.6
	L2	39 ± 4	3.74 ± 0.01	5.55 ± 0.06	5.37 ± 0.03	14.1	37.7	36.0	5.2	7.1
	L3	53 ± 1	5.3 ± 0.1	6.3 ± 0.1	6.65 ± 0.02	26.1	56.6	17.2	n.d	n.d
	L4	51 ± 2	5.85 ± 0.01	6.42 ± 0.01	5.32 ± 0.01	51.6	30.3	16.1	1.9	0.1
September 2009	L1	24 ± 1	2.3 ± 0.1	6.15 ± 0.05	3.31 ± 0.01	7.1	17.8	56.1	13.6	5.4
	L2	32 ± 3	3.92 ± 0.01	6.31 ± 0.01	3.84 ± 0.01	14.9	35.7	38.0	5.8	5.6
	L3	56 ± 2	5.3 ± 0.2	6.36 ± 0.02	9.56 ± 0.01	52.1	41.3	6.2	0.3	n.d
	L4	53 ± 1	5.98 ± 0.01	6.30 ± 0.02	8.0 ± 0.1	62.3	21.0	10.8	5.8	n.d
December 2009	L1	22 ± 1	1.85 ± 0.1	6.2 ± 0.1	0.24 ± 0.01	3.5	20.7	54.3	14.8	6.7
	L2	28 ± 1	2.90 ± 0.01	6.2 ± 0.1	1.15 ± 0.00	14.2	34.4	31.7	7.2	12.5
	L3	54 ± 1	5.1 ± 0.2	6.50 ± 0.01	3.55 ± 0.01	62.2	32.7	4.9	0.1	n.d
	L4	53 ± 1	5.70 ± 0.01	6.54 ± 0.02	2.60 ± 0.00	73.0	24.1	2.8	0.1	n.d

n.d - not detected

Rhizosediments collect at L1, the uppermost sampling site, had coarser particles, whereas sediments from L3 and L4 sampling site, from the lower estuary, had more than 80% of total particle size inferior to 0.25 mm. This trend was observed throughout the study.

Significant correlations were found between OM and Silt + Clay content (r = 0.920, $P < 0.05$, n = 15); water and Silt + Clay content (r = 0.897, $P < 0.05$, n = 15); and water and OM content (r = 0.987, $P < 0.05$, n = 15).

3.3.1.2 Total petroleum hydrocarbons concentration

Concentrations of TPH in rhizosediments varied between 0.032 and 0.535 mg $g^{-1}_{dry\ sediment}$ (Figure 3.1). TPH concentrations increased downstream, being, in general, significantly ($P < 0.05$) higher in L3 than in the uppermost sampling sites. In L4, TPH concentrations in rhizosediments were, in general, statistically similar ($P > 0.05$) to those found in L3. Considering the variation among seasons, although not significant ($P > 0.05$), TPH concentrations tended to decline in the period of high plant activity, rising during winter.

However in L3 rhizosediments, TPH concentration started increasing during autumn being values from December not different ($P > 0.05$) from those obtained in March. Moreover, significant ($P < 0.05$) positive correlations were found between TPH and Silt + Clay content (r = 0.56, p < 0.05, n = 15), and between TPH and OM content (r = 0.66, p < 0.05, n = 15).

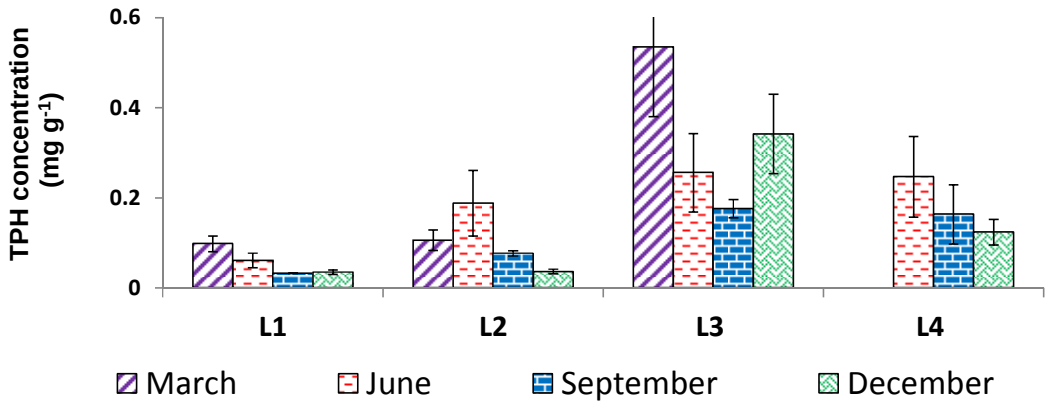

Figure 3.1 - Total petroleum hydrocarbons (TPH) concentrations (mg g^{-1}, mean and standard deviation, n = 3) in rhizosediments of *Juncus maritimus* (Sea Rush). Samples were collected at 4 different sampling sites (L1, L2, L3 and L4), and in four different seasons (in the beginning of March, June, September, December). In March, L4 rhizosediments were not sampled due to methodological/logistical problems.

3.3.1.3 Principal components analysis

To investigate further the relationship between the observed spatial distribution and temporal variation of samples and the rhizosediments characteristics (physical-chemical characteristics), PCA was applied to water and OM content, pH, EC, and grain size distribution of all seasons (Figure 3.2-A). PCA showed that the first principal component (PC1) axe, mainly related to the Silt + Clay, OM and water content (negatively) and with gravel, coarser and medium sand (positively), explained 73% of the variability. The second axe (PC2), characterized by pH (positively related), and EC and fine sand (negatively related), accounted for an additional 11.8% of the variability. Thus, both axes explained 84.8% of total variance.

PCA matrices revealed a clear pattern over the sampling sites, distributed along the PC1 gradient, with the upstream sites having coarser sediments and low OM content in the positive side, and the downstream sites with finer sediments and high OM content located on the negative side of the axis. Along the PC2 axis of variability, the separation of the sites was related to environmental factors (pH and EC) that seemed to be influenced by the river flow.

The PCA ordination with superimposition of hierarchical analysis of samples using Euclidean distances (Figure 3.2-B) showed three genuine clusters (I, II and III), obtained using the Simprof (similarity profile) permutation test (Figure 3.2-C). Clusters were mainly grouped according to their spatial distribution with significant ($P < 0.05$) differences between upstream and downstream sediments, indicating that the spatial distribution overhead the seasonal variability.

To investigate the relationship between the observed TPH concentrations and the prevailing environmental conditions, TPH concentrations were superimposed on the projection of samples (Figure 3.2-D). The size of the bubble reflects the TPH concentrations values, revealing a pattern along the PC1 gradient and the relationship with the environmental abiotic data grouping (Figure 3.2 - B). The general pattern revealed that higher TPH concentrations in the downstream estuary were associated with increased OM and Silt + Clay contents.

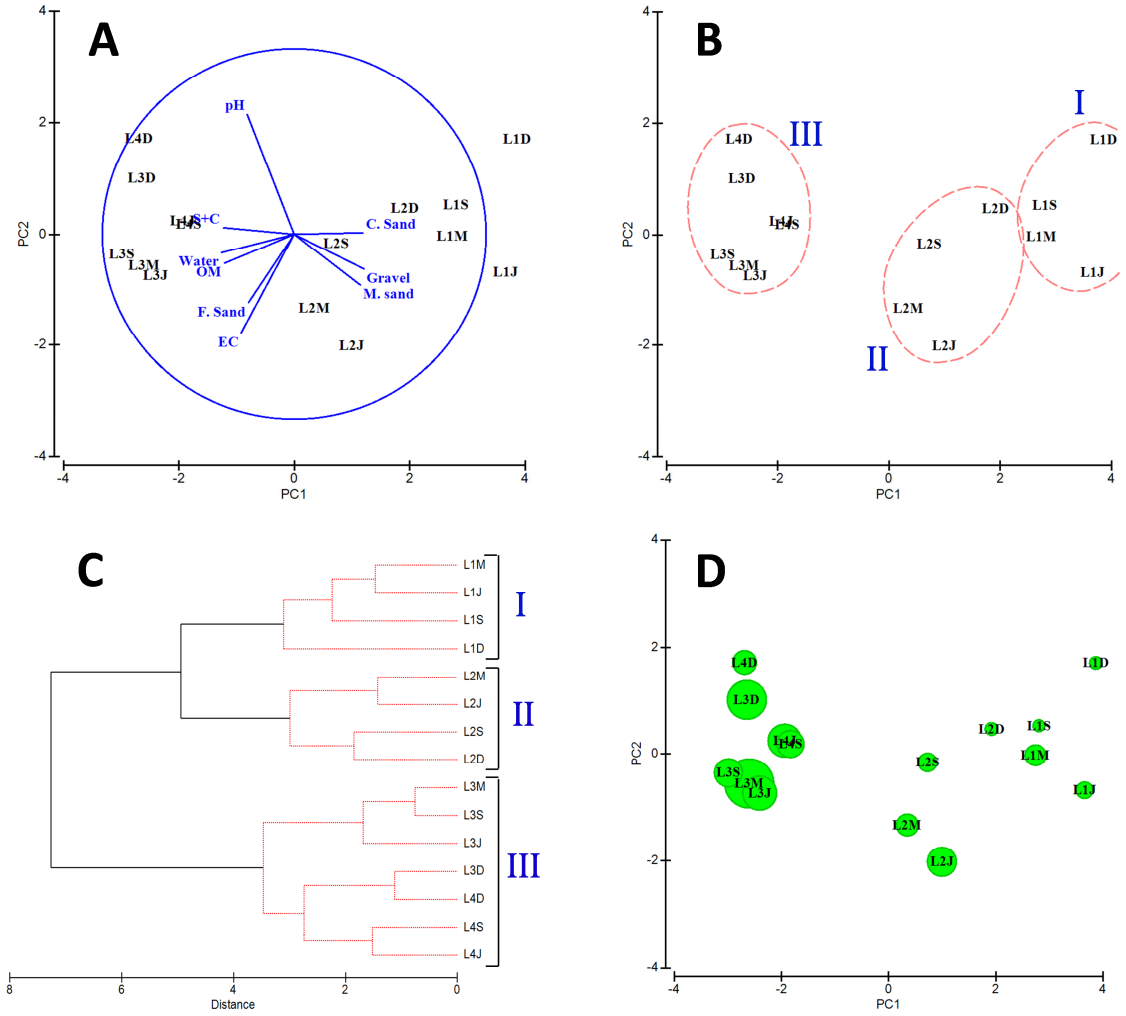

Figure 3.2 - **A)** Two dimensional principal component analysis (PCA) ordination based on the values of environmental abiotic data [Water and organic matter (OM) content, pH, electrical conductivity (EC) and grain size distribution]. **B)** PCA ordination with superimposition of genuine clusters (I, II and III), obtained using the Simprof (similarity profile) permutation test based on the lower triangular resemblance matrix created by using Euclidean distances. **C)** Dendogram generated from hierarchical analysis based in Euclidean distances grouping by average method, and using the Simprof test to verify differences between clusters generated. Bracketed samples encompass groupings (I, II and III) that are similar according to the Simprof test. D) PCA ordination with TPH concentrations superimposed on the projection of samples (the size of the bubble reflects the TPH concentrations value). Samples were collected at 4 different sampling sites (L1, L2, L3 and L4) in four different seasons [in the beginning of March (M), June (J), September (S) and December (D)]. In March, L4 rhizosediments were not sampled due to methodological/logistical problems.

3.3.2 Microbial analysis

3.3.2.1 Microbial abundance

The TCC abundance (Figure 3.3-A) and HD abundance (Figure 3.3-B) in rhizosediments in the four seasons were estimated. TCC ranged from 10^8 to 10^9 TCC $g^{-1}_{wet\ sediment}$, while HD ranged from 10^4 to 10^7 MPN $g^{-1}_{wet\ sediment}$.

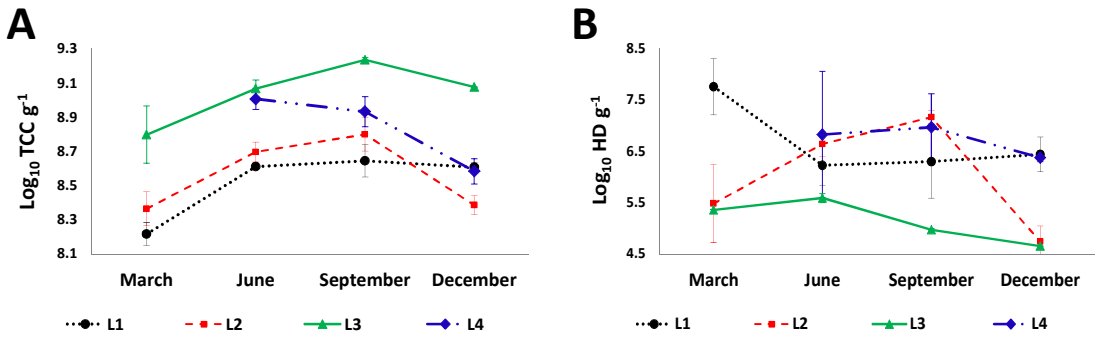

Figure 3.3 - **A)** Microbial abundance estimated by Total Cell Counts (log_{10} TCC g^{-1}, mean and standard deviation, n=3) in rhizosediments. **B)** Hydrocarbon degraders microorganisms (HD) estimated by most probable number (log_{10} HD g^{-1}, mean and standard deviation, n=2) in rhizosediments. *Juncus maritimus* (Sea Rush) rhizosediments were collected at 4 different sampling sites (L1, L2, L3 and L4) in four different seasons (in the beginning of March, June, September and December). In March L4 rhizosediments were not sampled due to methodological/logistical problems.

In general, TCC were significantly ($P < 0.05$) higher in L3 than in L1, L2 and L4 rhizosediments. TCC in L4 rhizosediments were also significant ($P < 0.05$) higher than L1 (in June, and September), and L2 (in June and December). No significant ($P > 0.05$) differences were found between TCC from L1 and L2 rhizosediments, except in December. Therefore, TCC tended to increase during the vegetative growth, flowering and fruiting phenological stages, *i.e.* in the seasons of higher plant activity (spring-summer), and to decline in the autumn, the senescent period. In addition, TCC were significant ($P < 0.05$) higher in June and September than in March (late winter). Significant ($P < 0.05$) correlations between TCC and OM ($r = 0.57$, $P < 0.05$, $n = 15$) and Silt + Clay fraction ($r = 0.57$, $P < 0.05$, $n = 15$) were observed. Considering only the

seasons of higher plant activity, stronger correlations were found with OM ($r = 0.84$, $P < 0.05$, $n = 8$), and with Silt + Clay fraction ($r = 0.78$, $P < 0.05$, $n = 8$).

In what HD abundance is concerned, seasonality seemed to promote significant ($P < 0.05$) differences in the HD rhizosediments, although a regular trend of variation was not observed. HD from L2 and L4 rhizosediment seemed to follow the trend observed in TCC, *i.e.* increase in the vegetative growth, flowering and fruiting phenological stages, and decline in December. However, HD in L1 and L3 rhizosediments showed different behaviors.

Due to this seasonal variation signal, comparison between the different sites in terms of rhizosediment HD was not straightforward. However, taken as a whole, HD from L1 and L4 were significantly ($P < 0.05$) higher than L3 rhizosediment, whereas L2 was either statistically identical or significantly higher than L3, depending on the season. Considering the three uppermost sampling sites, there was a significant ($P < 0.05$) negative correlation between HD and Silt + Clay content ($r = -0.681$, $P < 0.05$, $n = 12$).

3.3.2.2 Microbial community structure

Rhizosediment samples collected in September were chosen to assess the microbial community structure due to the higher total TCC found, and to the fact that spatial variability superimposed to the seasonal variability, as discussed in Section 3.3.1.3. ARISA analysis was performed in three replicates from each sample to evaluate shifts in the microbial community structure related to the differences of rhizosediments characteristics. Total number of peaks corresponded to different AFL, and thus to different bacterial phylotypes. Therefore, the most relevant information was given by the distribution of the different phylotypes in the different samples since it corresponds to differences in their genetic structure. The MDS plot (Figure 3.4) for all AFL showed that for each studied rhizosediment replicates were grouped together at 75% similarity, and well separate in the MDS ordering (the stress level was very low), suggesting good experimental replication. This indicates that they were more similar between each other than with any other sample, and Simprof test showed that microbial community structure was significantly ($P < 0.05$) different among rhizosediments. Moreover, microbial

community structure from downstream rhizosediments (L3 and L4) was more similar between them forming a subcluster at 50% similarity, showing dissimilarities with the upstream rhizosediments.

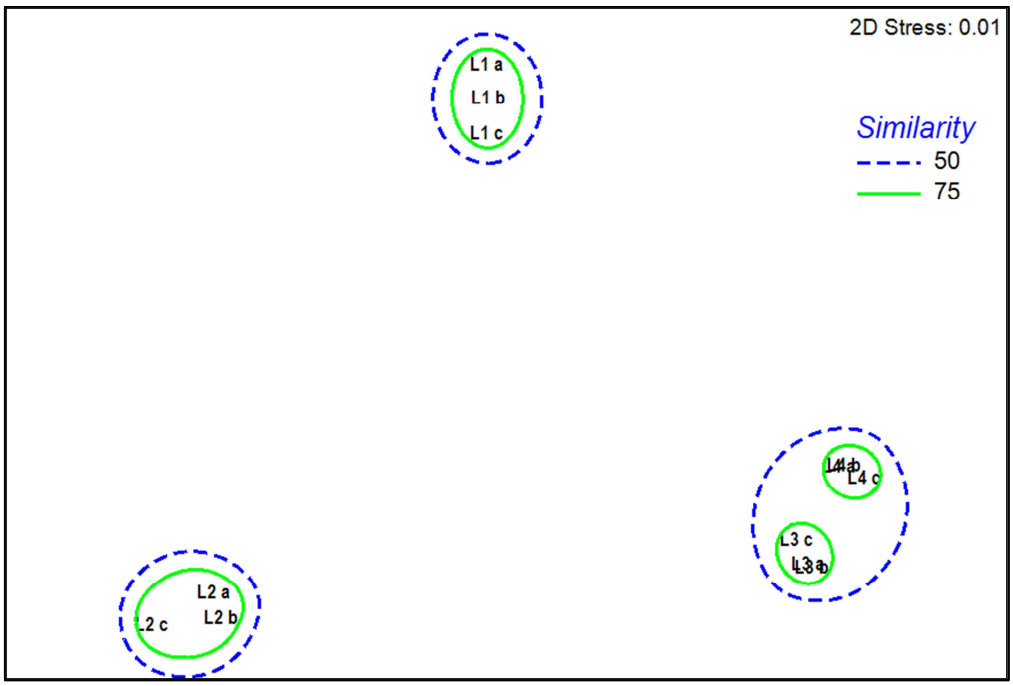

Figure 3.4 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities on presence/absence matrix obtained of ARISA fingerprints of microbial communities. Rhizosediments replicates were collected at 4 different sampling sites (L1, L2, L3 and L4) in the beginning of September 2009 in the Lima estuary. Replicates enclosed by circle clusters at 50% and 75% similarity.

3.3.3 Laboratory experiments

The characteristics of the 2010 batch of sediments, as well as the background levels of TPH and TCC were similar to those retrieved in the previous year, except for, HD counts. The yield was, with significantly (P < 0.05) higher counts in L1 than L2 and L4 rhizosediments in 2010; and with no differences (P > 0.05) among HD counts from L2, L3 and L4 rhizosediments.

Experiments without petroleum amendment (Table 3.2-A) yielded different TPH degradation rates, with higher rates in the upstream rhizosediments. Strong negative correlations between the percentages of degradation and OM (r = -

0.95, $P < 0.05$, n = 4) or Silt + Clay content (r = - 0.96, $p < 0.05$, n = 4) were found.

Table 3.2 - Total petroleum hydrocarbons (TPH) concentrations (mg g^{-1}, mean and standard deviation, n = 3) in rhizosediments of the batch slurry tests and percentage of TPH degradation observed after 15 days of incubation. A) Control experiments without petroleum amendment: concentrations in the background and after fifteen days (T_{15}) of incubation. B) Experiments with petroleum amendment: concentrations in the beginning (T_0) and after fifteen days (T_{15}) of incubation, with and without additional Nitrogen. Samples of *Juncus maritimus* rhizosediments were collected at 4 different sampling sites (L1, L2, L3 and L4) in summer (July - August) of 2010. Limit of detection (LOD): 0.032 mg g^{-1}

	A) Without petroleum amendment			***B) With petroleum amendment***				
					Without additional Nitrogen		With additional Nitrogen	
	TPH background concentration	T_{15} TPH concentration	Degradation	T_0 TPH concentration	T_{15} TPH concentration	Degradation	T_{15} TPH concentration	Degradation
L1	0.055 ± 0.004	<LOD	**88 %**	3.31 ± 0.46	2.67 ± 0.87	**19 %**	1.43 ± 0.77	**57 %**
L2	0.085 ± 0.025	0.037 ± 0.010	**56 %**	3.96 ± 0.63	2.57 ± 0.28	**35 %**		
L3	0.335 ± 0.057	0.332 ± 0.058	**1 %**	35.53 ± 2.56	24.74 ± 4.58	**30 %**	21.69 ± 7.59	**39 %**
L4	0.238 ± 0.086	0.192 ± 0.072	**19 %**	6.72 ± 0.07	5.70 ± 0.86	**15 %**		

Regarding total microbial abundance after 15 days of incubation, no significant ($P > 0.05$) differences were found in TCC comparing to the background levels. However, HD significantly ($P > 0.05$) increased, ca. 4 fold, and a strong positive correlation (r = 0.986, $P < 0.05$, n = 4) was found with HD background levels.

In experiments with petroleum amendment, the analysis of T_0 TPH (Table 3.2-B) showed differences between the several sediments, despite identical petroleum addition. Rhizosediments with the highest TPH background levels presented the higher TPH concentrations after petroleum amendment, reflecting different capacities of sediments to retain hydrocarbons. Although no significant ($P > 0.05$) correlation between the initial (T_0) concentrations and those in un-spiked rhizosediments was found, the Pearson coefficient (r = 0.85) was very strong. Moreover, experiments with petroleum amendment yielded different TPH degradation rates than experiments without petroleum amendment, with lower

rates in the upstream rhizosediments and a higher one in L3 rhizosediment. Considering rhizosediments with additional nitrogen, higher TPH degradation rates were found comparing with rhizosediments without additional nitrogen, with a degradation increase of 37% in L1 and of 9% in L3 rhizosediments. In the case of additional nitrogen, only two rhizosediments were tested due to methodological limitation, being chosen L1 and L3 rhizosediments as a result of their contrasting characteristics as shown above.

When petroleum was added to the medium, no correlation was found between percentages of TPH degradation and abiotic characteristics of the rhizosediments, unlike experiments without petroleum amendment. However, a strong positive correlation ($r = 0.99$, $P < 0.05$, $n = 4$) was found between initial (T_0) TPH concentrations and the amount of TPH degraded. Curiously, in experiments without petroleum amendment, the same variables were negatively related ($r = -0.83$), although the correlation was not significant ($P > 0.05$) due to sample size.

Finally, the HD abundance in the medium with petroleum amendment could not be quantified because of methodological saturation. Nevertheless, petroleum amendment yielded in general, significantly ($P < 0.05$) higher TCC comparing to both background levels and TCC after 15 days without petroleum amendment.

3.4 Discussion

Within the same environment, we found sediments with different characteristics (Table 3.1), despite the fact that they were colonized by the same plant, *J. maritimus*. Estuaries have strong gradients in many physical and chemical variables, as well as in the amount and composition of particles (Chapman and Wang, 2001). In fact, our study showed that most variables increased downstream. The PCA analysis (Figure 3.2-A) showed that rhizosediments particle size and OM content were the variables that mainly contributed to variations (PC1 explained 73% of total variance). Additionally, the cluster analysis (Figure 3.2-C) showed significant differences between upstream and downstream locations, grouping rhizosediments by sampling site, with the spatial variability overlaying the seasonal variability. The

superimposing of TPH concentrations on the projection of samples (Figure 3.2-D) suggest that rhizosediment characteristics within salt marshes can influence hydrocarbons distribution. Several studies (*e.g.* Amellal et al., 2001; Brunk et al., 1997; Chung and Alexander, 1999; Cunningham and Ow, 1996; Kottler et al., 2001; Rockne et al., 2002; Saghir et al., 2007; Talley et al., 2002; White et al., 1997) on un-colonized soil/sediments have shown a relationship between soil/sediments physical-chemical properties and the absorption, sequestration and bioavailability of diverse organic pollutants. Particularly, the OM content and small grain size particles have been considered to play a critical role in sorption/desorption and biodegradation processes (*e.g.* Bogan and Sullivan, 2003; Ghosh et al., 2000; Nam et al., 1998; Xia and Wang, 2008). Most of the organic matter resides in the small particle-size fraction (DeFlaun and Mayer, 1983; Kukkonen and Landrum, 1996), preventing the leaching of these particles (Badin et al., 2008), which explains the positive correlation found in this study between these two factors. Both factors have a constant negative charge that influences the adsorption of organic compounds (Otten et al., 1997), such as hydrocarbons. In fact, in this study, PCA analysis reflected the positive correlation found between OM content and Silt + Clay fraction and TPH concentrations and, therefore, their importance on hydrocarbons retention. But other factors must be considered, including one particularly important in estuarine areas, the salinity effect (Brunk et al., 1997). In fact, L3 and L4 rhizosediments were clustered together, both broadly characterized by high Silt + Clay and OM contents, but in one of the seasons they had different TPH concentrations, which could be explained by differences in salinity (measured as EC). In fact, with the increase of EC an increase in hydrocarbons sorption is expected (Brunk et al., 1997), and indeed, L3 had higher EC than L4.

The rhizosphere bacterial composition is likely to be affected by several factors, such as sediment characteristics (Bundy et al., 2002; Marschner et al., 2004; Sessitsch et al., 2001) that influence the growth, colonization, size and structure of bacterial populations. Indeed, the same outcome was found in this study, with differences among the microbial abundance (TCC) and the specific HD abundance, as well as, among the microbial community structures of the rhizosediments. Shifts on bacterial community composition were analyzed by ARISA, a DNA fingerprinting technique that allowed the rapid assessment of the genetic structure of complex communities in diverse environments

(Danovaro et al., 2009; Hewson and Furhman, 2004; Ranjard et al., 2001), and of the extent of changes caused by environmental disturbances (*e.g.* Malik et al., 2008). The MDS plot (Figure 3.4) based on the ARISA profiles revealed a clear division between the microbial community structure that inhabited the four sites. ARISA profiles from downstream rhizosediments were more similar between them forming a subcluster at 50% of similarity. Curiously, L3 and L4 sites were also clustered together by their rhizosediments characteristics (Figure 3.2-B). These data suggest that sediment characteristics probably contributed for the dissimilarity between microbial community structure of rhizosediments. This is in agreement with other studies in which the experimental data suggested that the soil characteristics were an important factor for determining rhizosphere community structure (Buyer et al., 1999; Latour et al., 1999; Marschner et al., 2001). It was, however, unclear from these studies, which soil properties contributed most, but Marschner et al. (2004) suggested that clay may exert a strong influence on rhizosphere community structures. In the present study, a higher TCC in the lower Lima Estuary (sites 3 and 4), when compared to the upper estuary, was observed, which can be related to the presence of finer estuarine sediments found in the lower area. Finer sediments provide a greater surface area to microbial colonization than coarse sediments, as well as higher stabilization (slow turn-over) of microbial cells and a more protective habitat through pore size exclusion of predators (Chenu and Stotzky, 2002; DeFlaun and Mayer, 1983; England et al., 1993; Kabir et al., 1994; Sessitsch et al., 2001). In fact, several studies (Dale, 1974; Kabir et al., 1994; Kanzog and Ramette, 2009) also reported the increase of total microbial abundance in fine-grained particles. However this effect may also correspond to an indirect relation with the OM content. As previously mention, most of OM resides in the small particle-size fraction (Kukkonen and Landrum, 1996), and, in fact, sediments in sites L3 and L4 were also richer in OM when compared with the upper sites. van Gestel et al. (1996) have demonstrated that both clay and organic carbon contributed to quantitative microbial biomass distribution in a silty loam soil. Small particles and organic colloids seem to ensure microbial growth and survival by their capacity to buffer the nutrient supply, water availability and source of carbon substrate (Ranjard and Richaume, 2001).

Actually, we found positive correlations between Silt + Clay and OM content and microbial abundance, an outcome in agreement with other studies in un-colonized sediments (Blum et al., 2004; Kanzog and Ramette, 2009; Köster et al., 2005; Llobet-Brossa et al., 1998; Yamamoto and Lopez, 1985). These correlations were stronger in seasons of higher plant activity, spring and summer (June and September sampling), probably due to the plant influence. In fact, despite the differences among rhizosediments TCC, we found a similar seasonal trend for this parameter at all sites, with an increase on microbial abundance during June and September. This could be related to the phenological behavior of plants. Spring and summer are the seasons of higher plant activity, generally with higher root exudation (Palomino et al., 2005), creating the so-called "rhizosphere effect" (Olson et al., 2003), which can enhance the microbial growth in the rhizosphere. Temperature is also linked to changes in soil microbial communities (Palomino et al., 2005; Waldrop and Firestone, 2006), and several studies (*e.g.* DeFlaun and Mayer, 1983; Shiah and Ducklow, 1994) demonstrated that temperature also influence this seasonal behavior. The seasonal cycle of estuarine bacteria can be interpreted as a physiological response of the bacterial metabolic rates, reproductive and growth rate, to the "*in situ*" temperature and to the phenological plant activity (DeFlaun and Mayer, 1983; Ribeiro et al., 2013a).

Curiously, seasonality also seemed to promote significant ($P < 0.05$) differences in the HD among rhizosediments. However, no regular trend of variation among the different rhizosediments could be observed. In a separate study from the authors (Ribeiro et al., 2013a) the above mentioned "rhizosphere effect" enhanced microbial growth when compared to un-colonized sediments. Nevertheless, the increased temperature during spring and summer seemed to be the main factor that influenced HD abundance during the phenological cycle. Despite this fact, we found differences among HD counts from the different rhizosediments, pointing to the sediments characteristics influence. Contrasting with TCC, the L3 site presented the lower HD abundance, and L1 site showed higher counts. This outcome suggested that coarser particles can provide more favorable conditions for the growth of HD. In fact, Amellal et al. (2001) also found a high density of HD in aggregates corresponding to coarser sediments, suggesting a key influence of this factor. Moreover, when considering only the three upper sites (L1, L2 and L3), a

negative correlation was found between HD abundance and Silt + Clay content. We hypothesize that coarser textured sediments with a granular structure provided more favorable conditions for the growth of aerobic pollutant degrading microorganisms. On one hand, during tidal exposure, fine textured and compressed sediments are likely to reduce the exchange of gases with the atmosphere, restricting oxygen diffusion through sediments to microorganisms located in the aggregates (Bradley and Morris, 1990; Horn et al., 1994; Kabir et al., 1994), and limiting the proliferation of aerobic HD, the most predominantly hydrocarbon degraders isolated from contaminated ecosystems (Chaillan et al., 2004; Head et al., 2006; Rosenberg, 2006). On the other hand, hydrocarbons can be more accessible and available in sand aggregates than on clay particles (Brady and Weil, 1996; Otten et al., 1997), and the lower OM content in coarser sediments may restrict OM competition with hydrocarbons as a carbon source (Slater et al., 2005), fostering the HD abundance. These reasons could explain why at site L3, the location with the higher Silt + Clay and OM content, a lower HD abundance was observed despite the high TPH concentrations. However, the fact that L3 and L4 rhizosediments presented similar OM and Silt + Clay content but divergent HD abundance, suggested that other factors could also play an important role in HD community.

The evidences described above suggested that rhizosediments characteristics could influence rhizosphere bacterial composition as well as the distribution and bioavailability of hydrocarbons with consequences for the rhizosphere hydrocarbon degradation microbial potential. The laboratory experiment seemed to confirm this hypothesis. Experiments without petroleum amendment yielded higher TPH degradation rates in the coarser rhizosediments. With a strong negative correlation, Silt + Clay and OM content seemed, once again, to be the most influent factors in the degradability of hydrocarbons. Our findings were consistent with other soils studies (Carmichael and Pfaender, 1997; Frick et al., 1999 and the reference therein; White et al., 1997;), that also have showed a lower rate of mineralization of hydrocarbon in smaller aggregates; highlighting that soils with high organic carbon content (> 5%) usually lead to strong adsorption and, therefore, low availability, while a moderate organic carbon content (1–5 %) may lead to limited availability. In fact, despite the high TPH concentrations, L3 site

displayed the lower hydrocarbons degradation, which can be related to the low bioavailability, inherent to sediments with high Silt + Clay and OM content (Amellal et al., 2001; White et al., 1997). Some studies (*e.g.* Eyvazi and Zytner, 2009) also found a similar correlation between hydrocarbon degradation rates and soil properties, particularly, Silt and Clay content. Moreover, the initial population of HD was considered by Eyvazi and Zytner (2009) a major factor affecting the bioremediation rate. In this study, no relationships between initial population of HD and the initial TPH concentrations, nor with hydrocarbon degradation rates were found. We hypothesize that microbial communities in L3 rhizosediment were not metabolizing hydrocarbons residues at a high pace, suggesting a cometabolic process where the HD are growing on other carbon sources, such as OM, an outcome found in salt marshes by Slater et al. (2005). On the other side, despite the lower TPH of L1 rhizosediments, TPH degradation was significantly ($P < 0.05$) higher, which is probably related to higher hydrocarbon availability, since this site had coarser sediment and lower OM content. Therefore, HD were probably metabolizing hydrocarbons as carbon source.

Laboratory experiments with petroleum amendment yielded degradation rates with small differences among rhizosediments, opposing to the verified in experiments without petroleum amendment. The T_0 TPH concentrations, once again, reflected the different capacities to retain hydrocarbons after the amendment of petroleum, with TPH concentrations in finer sediments being higher by over an order of magnitude compared to the coarser sediments. A similar assumption was previously reported by Scherr et al. (2007) for soils. However, rhizosediment characteristics seemed to have less influence on TPH degradation rates of recently spiked hydrocarbons, probably because in this case hydrocarbons were equally bioavailable. Even at L3 rhizosediment, where hydrocarbons degradation was not noteworthy in experiments without petroleum amendment, showed reasonable TPH degradation.

Although all experiments had BH medium, a mineral-salts enrichment, TPH degradation rates in experiments with additional nitrogen increased, showing that nutrients such as nitrogen become the limiting factor for microbial degradation after heavy contamination, an outcome also reported in other studies (*e.g.* Zhou and Crawford, 1995). Pearson et al. (2008) has demonstrated that a salt marsh microbial community responded rapidly to the

introduction of hydrocarbons, and after two weeks, up to 26% of bacterial biomass was derived from consumption of the freshly spilled oil. In fact, we verified that microbial abundance increased significantly ($P < 0.05$) in rhizosediments with petroleum amendment, inclusively leading to the methodological saturation of the HD MPN.

3.5 Conclusion

This study represents the first insight into the influence of natural sediment characteristics, namely Silt + Clay and OM content, on the hydrocarbon degradation potential of salt marsh microbial communities. In fact, higher Silt + Clay and OM content tended to increase the retention of hydrocarbons around *J. maritimus* belowground tissues. On the other hand, our study also showed that the rhizosediments characteristics can influence the microbial structure. Despite Silt + Clay and OM content favored the increase of the total microbial abundance, these parameters were associated to the decrease of hydrocarbon degraders probably due to lower hydrocarbon bioavailability. Moreover, laboratory experiments confirmed that higher Silt + Clay and OM content could influence negatively TPH degradation rates. Therefore, this study presents a valuable outcome to the evaluation and management of rhizoremediation technology, with wider applicability in estuarine ecosystems. This is the first insight into the influence of natural sediment characteristic on the hydrocarbon degradation potential of salt marsh microorganisms, a feature that should be considered when designing rhizoremediation strategies in estuaries.

Chapter 4

Influence of different salt marsh plants on hydrocarbon degrading microorganisms abundance throughout a phenological cycle

Published in International Journal of Phytoremediation, 2013
Volume 15, 715-728

Influence of different salt marsh plants on hydrocarbon degrading microorganisms abundance throughout a phenological cycle

4.1 Introduction

Salt marsh areas have an important ecological role since are among the most productive ecosystems on Earth (Boorman, 1999). In these areas, the presence of vegetation can influence the microbial community (Hartmann et al., 2009; Kirk et al., 2005), through sediment oxygenation (Williams et al., 1994), the release of a range of organic compounds, such as root exudates, and eventually through nutrients release when the roots die and are degraded (Kuiper et al., 2004; Olson et al., 2003; Salt et al., 1998). The interactions between plants and microorganisms in the rhizosphere are complex and varied (Lambers et al., 2009), being influenced by the plant species involved. Environmental conditions (*e.g.* temperature, humidity, flooding regime, salinity), and the presence of plant species with different growing patterns, resource allocation and root exudates could lead to different populations or different degrees of microbial activity (Burke et al., 2002; Palomino et al., 2005). In fact, the rhizosphere is an ideal microhabitat for increasing the number of microorganisms (Hutchinson et al., 2003), including hydrocarbon-degrading microbial population in salt marshes (Ribeiro et al., 2011).

PHC are among the most common contaminants bound to estuarine sediments (Chapman and Wang, 2001), giving rise to significant environmental concern (Daane et al., 2001). Discharges from municipal and industrial wastewater, urban runoff and oil leakage from boats and ships are possible sources of hydrocarbon contamination. Salt marshes are extremely sensitive to pollutants, including oil pollution (Andrade et al., 2004). Therefore, it is important to clean and recover these areas, which can be a difficult task (Zhu et al., 2004). The use of plants and their associated microorganisms for the treatment of contaminated media is referred to as rhizodegradation or rhizoremediation (Olson et al., 2003; Gaskin and Benthan, 2010). One of the main drawbacks of

rhizoremediation is the need of identifying plant species with potential to promote hydrocarbon degradation (Gaskin et al., 2008; Kulakow et al., 2000). Therefore, the improvement of performance and acceptance of cost-saving bioremediation techniques is, thus, a major challenge (Juwarkar et al., 2010). In soils, the presence of vegetation can accelerate the bioremediation of areas contaminated with petroleum hydrocarbons (Tischer and Hübner, 2002), supporting microorganisms to yield less toxic, less mobile and/or less bioavailable products (Vidali, 2001). Although hydrocarbon rhizoremediation in soils has been widely addressed, studies on salt marshes sediments are scarce.

Having in mind the need to increase the scientific knowledge for the development of alternative, and cost saving approaches to tackle coastal oil pollution, as the recent oil spill in the Gulf of Mexico highlighted, it is still not clear how and to which extent salt marsh plants may influence hydrocarbon degrading microbial communities and, therefore, petroleum hydrocarbon degradation (Daane et al., 2001). The knowledge of rhizoremediation potential of plants "*in situ*", and how seasonality affect this potential is an important requirement for the development of appropriate management strategies, as well as for the implementation of long-term rhizoremediation techniques in salt marshes.

In this vein, the present study aimed to assess the rhizoremediation potential, particularly the eventual fostering of hydrocarbon degrading microorganisms by different salt marsh plants, when compared with un-colonized sediments located at the vicinity of the plants. Additionally, the variability of the rhizoremediation activity potential during a year-long plant life cycle was also studied.

4.2 Materials and Methods

4.2.1 The study area

The Lima River Estuary is the end member of an international watershed located in NW Portugal, an urban-industrialized water body with a large salt marsh area. The selected sampling site, L3 (Figure 2.1), located in the lower estuary with salinity ranging from 5 to 35, was colonized by individual

assemblages of *Juncus maritimus*, *Phragmites australis*, and *Triglochin striata* (ca. 2 m from each other). These plants are perennial, belonging to different families: *J. maritimus* (native) belongs to the *Juncaceae* family, flowering in June-September; *P. australis* (invasive) belongs to the *Gramineae/Poaceae* family, flowering in July-September; *T. striata* (exotic) belongs to the *Juncaginaceae* family, flowering in May-June (Coutinho, 1913; Sampaio, 1988; Franco and Afonso, 1994; 1998; 2003). *J. maritimus* and *P. australis* are common examples of ubiquitous species in the Portuguese salt marshes (Costa et al., 2009a), whereas *T. striata* is native from the Austral-Asian, South African and American territories, and its occurrence in Portugal is restricted to the north-western coast (Costa et al., 2009b), with patches being observed in the studied marsh site. *J. maritimus*, the only plant with an appreciable rhizome structure, presented adventitious roots borne on the horizontal rhizome, generally, with thicker roots than the other plants. *J. maritimus* does not present a live/death cycle with belowground (roots and rhizomes), and aboveground structures being quite stable during a phenological cycle (Almeida et al., 2006). In fact, individual plants died but simultaneously, being replaced by a new batch of plants. On the other hand, *P. australis* has a fibrous and dense root system, that growths vigorous in spring and autumn, while the rhizome revealed intense growth in summer (Engloner, 2009 and references therein), presenting shorter aboveground tissues during late autumn and winter. Just like *J. maritimus*, *P. australis* does not seem to have a live/death cycle in the Lima estuary. As for *T. striata*, most of its biomass is located belowground, in a fibrous and dense root system. According to Laegdsgaard (2006), in dry conditions aboveground tissues die down leaving belowground rhizomes. In the Lima estuary, this senescence behavior, with the disappearing of most aboveground tissues, was observed in autumn.

In the Lima River estuary, the belowground biomass (dry) of each plant, estimated from 10 cm long, 10 cm large and 20 cm depth sediment plots, was 2.03 g dm^{-2} for *J. maritimus*, 4.13 g dm^{-2} for *P. australis* and 16.42 g dm^{-2} for *T. striata* (Almeida et al., 2011). Roots and rhizomes biomass (dry) accounted, respectively, for 5% and 17% of plant total weight in *J. maritimus*, for ca. 17% and 60% in *T. striata*; and for ca. 8% and 6% in *P. australis*. The remaining mass was aboveground tissues (Almeida et al., 2011).

4.2.2 Sediment sampling and characterization

Sediment samples were collected in late winter (March), spring (June), summer (September), and autumn (December) of 2009. Sub-surface sediments un-colonized and colonized (rhizosediments) were collected into sterile plastic bags. All sediments were retrieved between 5 and 15 cm, the depth with the higher plant belowground biomass in the case of colonized sediments. Further details on transport of samples, determination of water and OM content, as well as particle size distribution can be found in Ribeiro et al. (2011).

4.2.3 Enumeration of Microorganisms

TCC were obtained by DAPI direct count method (Porter and Feig, 1980).

HD were estimated using a modified MPN protocol (Wrenn and Venosa 1996) in 96-well microtiter plates. Further details can be found in Ribeiro et al. (2011).

4.2.4 Determination of total petroleum hydrocarbons concentration

Prior to TPH analysis, sediment samples were dried at room temperature until constant weight and sieved through a nylon net of 2 mm mesh to remove large particles and loosen roots. For TPH measurements, a previously optimized method (Couto et al., 2012) was used for the sediment samples.

4.2.5 Statistical and data analysis

The mean and standard deviation values were calculated. Microbial enumeration data were normalized by logarithm ($\log_{10}$) transformation prior to statistical analysis. Significant ($P < 0.05$) differences among TCC, HD, TPH, and OM were analyzed by a parametric one-way ANOVA. If any significant difference was detected, a Tukey test for unequal sample sizes was used to determine which means were significantly different. Correlation factors ($P < 0.05$) were analyzed by correlation matrices. All statistical tests were performed using the commercial software STATISTICA (data analysis software system), version 7. StatSoft, Inc. (2004).

4.3 Results

4.3.1 Sediment characterization

Un-colonized sediments and rhizosediments of *J. maritimus*, *P. australis*, and *T. striata* were characterized in terms of water and OM content as well as grain size distribution (Table 4.1). Independently of the presence of plants, sediments were dominated by small grain size particles, with over 80% of total particle size being <0.25 mm.

Table 4.1 - Water (H_2O) and organic matter (OM) contents (in percentage) (mean and standard deviation, n = 3) and particle size fractions of un-colonized sediments (Sed) and rhizosediments (Rhizo) of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata*. Samples were collected in four different seasons [late winter (March), spring (June), summer (September) and autumn (December)] in 2009 at L3 sampling site.

Sample		% H_2O	% OM	Particle size fraction percentage relatively to total weight				
				Silt + Clay	Fine sand	Medium sand	Coarse sand	Gravel
March	Un-colonized Sed	51 ± 1	5.3 ± 0.5	50.6	23.3	25.8	0.4	n.d
	Rhizo *J. maritimus*	50.3 ± 0.8	5.53 ± 0.09	49.3	40.3	10.3	0.1	n.d
	Rhizo *P. australis*	56.8 ± 0.8	5.7 ± 0.1	60.5	32.9	6.5	0.1	n.d
	Rhizo *T. striata*	53.2 ± 0.4	6.30 ± 0.02	70.3	29.0	0.7	n.d	n.d
June	Un-colonized Sed	50 ± 1	5.4 ± 0.2	49.7	34.6	15.5	0.2	n.d
	Rhizo *J. maritimus*	53 ± 1	5.3 ± 0.1	26.1	56.6	17.2	n.d	n.d
	Rhizo *P. australis*	62 ± 1	6.00 ± 0.05	50.4	35.7	13.9	n.d	n.d
	Rhizo *T. striata*	53.2 ± 0.4	6.4 ± 0.2	40.8	53.2	6.0	n.d	n.d
September	Un-colonized Sed	50.6 ± 0.6	4.74 ± 0.07	58.6	28.2	13.2	n.d	n.d
	Rhizo *J. maritimus*	56 ± 2	5.3 ± 0.2	52.1	41.3	6.2	0.3	n.d
	Rhizo *P. australis*	60.4 ± 0.1	5.31 ± 0.02	67.0	26.7	6.3	n.d	n.d
	Rhizo *T. striata*	51.7 ± 0.8	6.26 ± 0.08	65.5	33.7	0.8	n.d	n.d
December	Un-colonized Sed	53.6 ± 0.8	4.76 ± 0.03	58.4	29.0	12.5	0.1	n.d
	Rhizo *J. maritimus*	54.0 ± 0.5	5.1 ± 0.2	62.2	32.7	4.9	0.1	n.d
	Rhizo *P. australis*	64.7 ± 0.7	5.7 ± 0.2	63.4	34.8	1.7	0.1	n.d
	Rhizo *T. striata*	48.5 ± 0.1	5.7 ± 0.1	71.4	27.6	1.0	0.3	n.d

n.d: not detected

In general, rhizosediments showed higher water and OM contents when compared with un-colonized sediments. On the other hand, *P. australis* rhizosediment showed a significantly ($P < 0.05$) higher water content, whereas,

T. striata rhizosediment had significantly ($P < 0.05$) higher OM content compared to the others. Seasonality seemed to promote significant ($P < 0.05$) differences in the sediments water and OM contents, although a regular trend of variation was not observed.

4.3.2 Total petroleum hydrocarbons concentration

The concentration of TPH in un-colonized sediments and rhizosediments are presented in Figure 4.1.

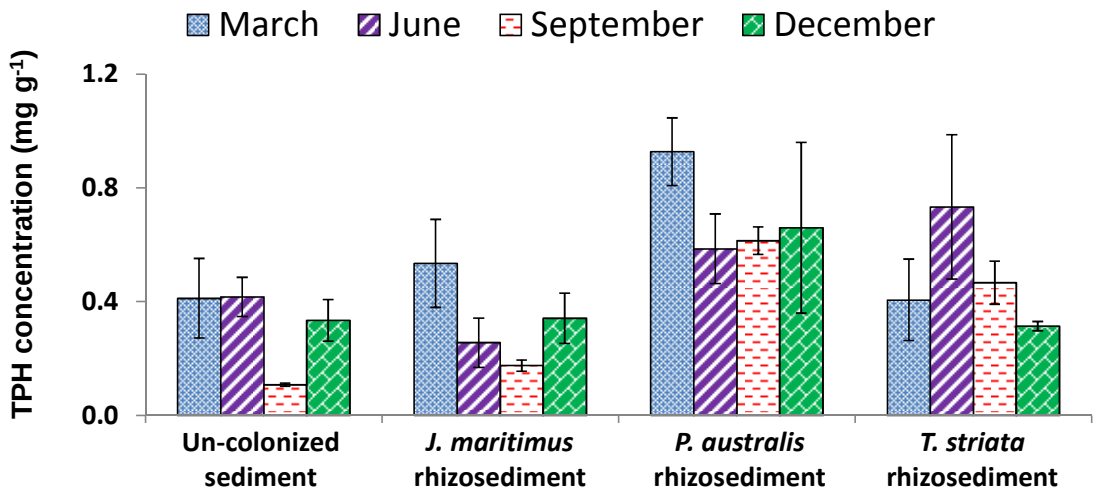

Figure 4.1 - Total petroleum hydrocarbons (TPH) concentrations (mg g^{-1}, mean and standard deviation, n = 3) in un-colonized sediments and rhizosediments of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata*. Samples were collected in four different seasons [late winter (March), spring (June), summer (September) and autumn (December)] in 2009 at L3 sampling site.

In *P. australis* rhizosediments, TPH concentrations were always higher than those in un-colonized sediments, being the differences significant ($P < 0.05$) only in winter and summer. The TPH concentrations in *T. striata* were significantly ($P < 0.05$) higher than those in the surrounding un-colonized sediments, but only in spring and summer, the seasons of higher plant activity.

On the other hand, the TPH concentrations in *J. maritimus* rhizosediments were similar ($P > 0.05$) to those in the surrounding un-colonized sediment.

Important differences were also found between rhizosediments of the different plants. TPH concentrations were significantly ($P < 0.05$) higher in *P. australis* rhizosediments, than in *J. maritimus* and *T. striata* colonized areas, particularly in winter, summer and spring (only for *J. maritimus*). Moreover, in the seasons of higher plant activity, TPH concentrations in *T. striata* rhizosediments were significantly ($P < 0.05$) higher than *J. maritimus* rhizosediments.

Except for *T. striata* rhizosediments, a general trend for TPH concentration to decline during the seasons of higher plant activity, and a rise up in the senescent stage.

4.3.3 Microbial Counts

4.3.3.1 TCC abundance

In general, a significantly ($P < 0.05$) higher TCC in rhizosediments compared to un-colonized sediments was observed, being the only exception winter, when no significant ($P > 0.05$) differences could be found (Figure 4.2).

No significant ($P > 0.05$) differences between rhizosediments were found, but in the case of *T. striata* during the autumn season, when TCC abundance was higher ($P < 0.05$). In un-colonized sediments, no significant ($P > 0.05$) differences were found in TCC throughout the year.

Generally, there was a trend for TCC in *J. maritimus* and *P. australis* rhizosediments to increase in the vegetative growth, flowering and fruiting phenological stages, *i.e.* in the seasons of higher plant activity, followed by a decline in autumn. However, *T. striata* showed a different behavior with a continuous TCC increase after the summer, *i.e.* during the senescence phenological stage of the plant.

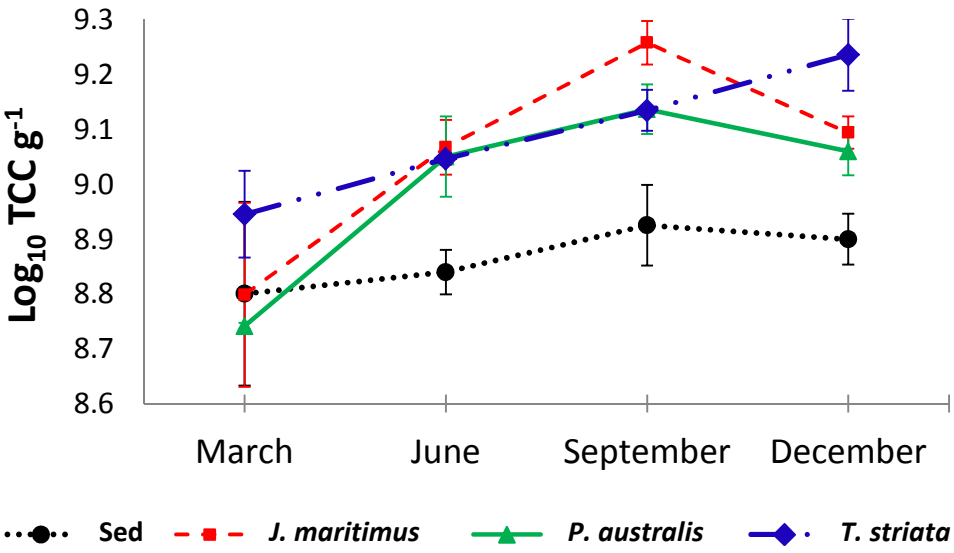

Figure 4.2 - Variation of microbial abundance estimated by total cell counts (log_{10} TCC g^{-1}, mean and standard deviation, n = 3) in un-colonized sediments (Sed) and rhizosediments of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata* through one year. Samples were collected in four different seasons [late winter (March), spring (June), summer (September) and autumn (December)] at L3 sampling site.

4.3.3.2 HD microorganisms abundance

Considering all seasons, only the *T. striata* rhizosediments HD abundances were significantly ($P < 0.05$) higher than those in un-colonized sediments (Figure 4.3). *P. australis* showed a similar although not significant ($P > 0.05$) behavior. Between rhizosediments, the abundance of HD in *T. striata* rhizosediments was always significantly ($P < 0.05$) higher than in *J. maritimus* rhizosediments and significantly ($P < 0.05$) higher than in *P. australis* rhizosediments in summer and autumn. Although HD counts were higher in *P. australis* rhizosediments, no significant ($P > 0.05$) differences were found between *P. australis* and *J. maritimus* rhizosediments HD abundance.

In un-colonized sediments, HD were significantly ($P < 0.05$) higher in spring than in winter and autumn. The same trend was observed for *P. australis* and *J. maritimus* with HD abundance being higher in spring, during the vegetative growth, before the flowering stage (July-September). Again, the HD abundance variation in *T. striata* rhizosediment was different from the other two plants,

being only significantly ($P < 0.05$) lower in winter. The higher HD abundance in the *T. striata* rhizosediment was observed during the flowering period (May-June) and the following fruiting stage (season of higher plant activity), concomitantly during the senescence phenological stage, no differences ($P > 0.05$) were observed. Generally, there was a regular pattern of variation of HD abundance, increasing during the spring, vegetative growth and flowering stage of plants.

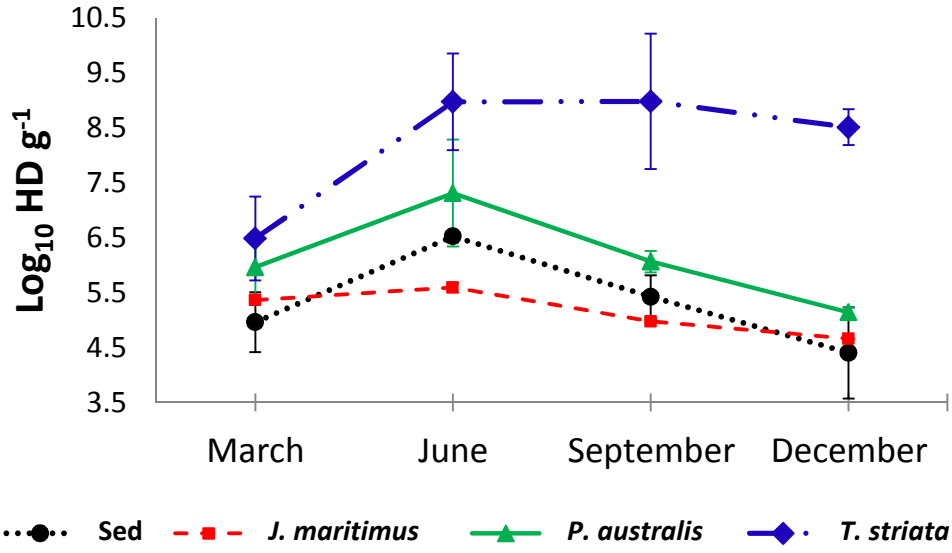

Figure 4.3 - Variation of Hydrocarbon Degraders microorganisms estimated by Most Probable Number (log_{10} HD g^{-1}, mean and standard deviation, n = 2) in un-colonized sediments (Sed) and rhizosediments of *Juncus maritimus*; *Phragmites australis*; *Triglochin striata* through one year. Samples were collected in four different seasons [late winter (March), spring (June), summer (September) and autumn (December)] at L3 sampling site.

4.3.4 Correlations factors

Correlation factors ($P < 0.05$) between TPH concentrations and both abiotic (OM) and biotic (HD microorganisms) parameters were assessed. Having all seasons into account, a positive and significant correlation between TPH vs. OM ($r = 0.53$, $P < 0.05$, $n = 16$) emerged (Figure 4.4-A). However, no relationship on TPH vs HD was found (Figure 4.4-B). Considering only the

seasons of high plant activity (spring and summer), besides the significant correlation on TPH vs OM (r = 0.76, $P < 0.05$, n = 8) (Figure 4.4-C), a significant correlation on TPH vs HD (r = 0.74, $P < 0.05$, n = 8) was also obtained (Figure 4.4-D).

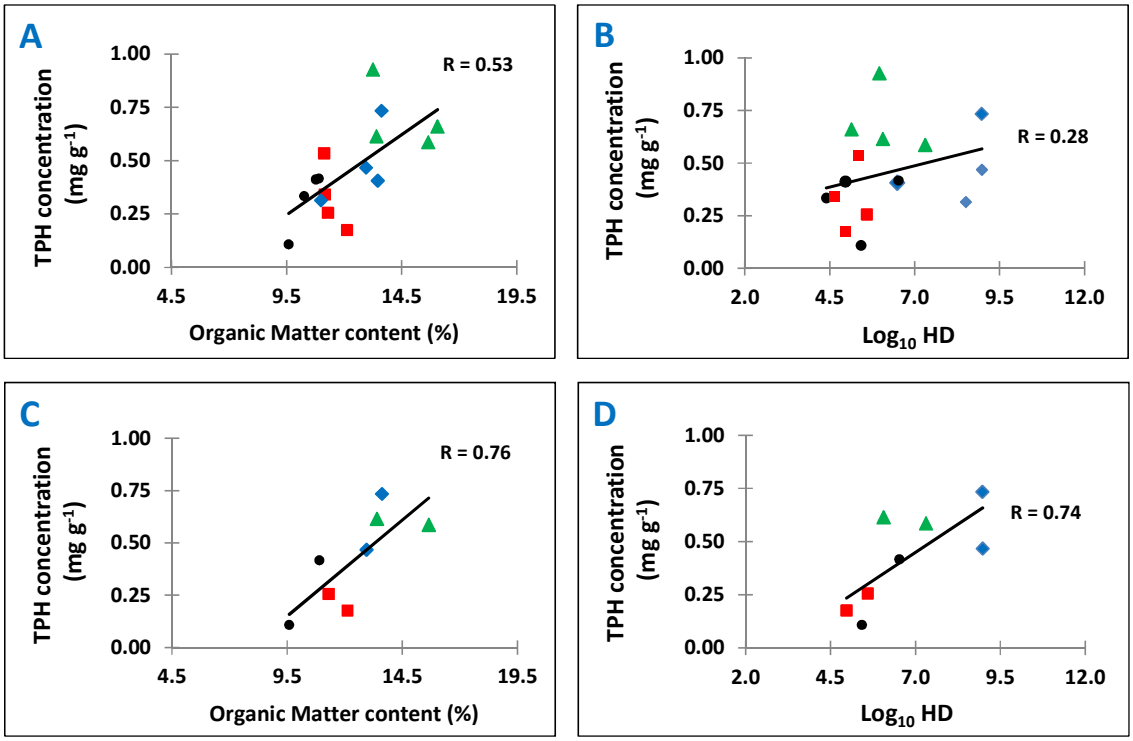

Figure 4.4 – **A)** Correlation plot of total petroleum hydrocarbons (TPH) concentrations vs organic matter content considering all seasons. **B)** Correlation plot of TPH concentrations vs hydrocarbon degraders (HD) microorganisms, estimated by Most Probable Number (Log_{10} HD), considering all seasons. **C)** Correlation plot of TPH concentrations vs organic matter content considering the seasons of high plant activity. **D)**. Correlation plot of TPH concentrations vs HD considering only the seasons of high plant activity. Simbols represents un-colonized sediments (●); and colonized sediments by *Juncus maritimus* (■); *Phragmites australis* (▲); *Triglochin striata* (◆).

4.4 Discussion

In estuaries, TPH contamination along the system can be variable, because sediments may have considerably different capacities for collecting contaminants (Chapman and Wang, 2001). The organic carbon fraction has

been identified as the most important factor for the control of the concentrations of organic contaminants like hydrocarbons (Chapman and Wang, 2001). Our study demonstrated significant correlations ($P < 0.05$) between TPH and OM content, an outcome also reporter by Paixão et al. (2011) in mangrove sediments. Plants are a major source of organic matter into sediments because root exudates can polymerize with humic materials to form large and stable aggregate structures that are conducive to sequestration of organic carbon, which rises OM content and, consequently, increasing the binding of hydrocarbons (Gregory et al., 2005). Our results showed that the rhizosediments of *T. striata* and *P. australis* had not only higher OM but also higher TPH concentrations than the surrounding un-colonized sediment. Similar findings were reported by Martins et al. (2008), which observed an extremely higher incorporation of hydrocarbons in a salt marsh rhizosediment than in un-colonized sediments. The significant ($P < 0.05$) higher concentrations of TPH in rhizosediments found in our study may be explained by the movement of compounds towards the roots, due to the plant uptake of water and dissolved nutrients, from the surrounding sediments (Clothier and Green, 1997), followed by the subsequent sequestration onto the OM, as discussed previously. Interestingly, *P. australis* rhizosediments had not only the highest water contents, but also the highest TPH concentrations, suggesting the probable movement of compounds due to the plant uptake of water.

The TPH concentrations in rhizosediments were significantly higher than in uncolonized sediment during spring and summer. A higher uptake of essential nutrients in spring and summer due to the higher plant activity (additional hours of sun light and increased temperatures), with a higher movement of compounds within sediments, has been reported (Almeida et al., 2006). Therefore, our results clearly highlight the role of plants on the movement of hydrocarbons, with a greater yield during the higher activity seasons. Moreover, the TPH concentrations in *J. maritimus* rhizosediments were significantly ($P < 0.05$) lower than in *P. australis* and *T. striata* rhizosediment in spring and summer, pointing to the influence of the root morphology and distinct patterns of resource allocation of different plants, as indicated by Burke et al. (2002), but also to the seasonal variability of this allocation. It must be stressed that introduced salt marsh plants, like *P. australis* and *T.*

striata, with fibrous and dense root morphology, presented higher belowground biomass per soil volume than native plants, such as *J. maritimus*, with adventitious root system (Almeida et al., 2011).

The loss of carbon compounds from roots is a key process for the development of enhanced microbial populations in the rhizosphere when compared with the un-colonized soil (Morgan et al., 2005). The present study demonstrated, with the exception of winter, a significantly higher total microbial abundance in rhizosediments comparatively to uncolonized sediment (in general, an order of magnitude of difference), which was consistent with results reported in other studies performed in soil rhizosphere (*e.g.* Ho and Banks, 2006; Muratova et al., 2003). This was presumably because plants roots release oxygen, carbon and nitrogen compounds, especially small molecules such as amino acids, sugars and organic acids, creating a nutrient-rich environment in which microbial activity was stimulated (Kuiper et al., 2004; Salt et al., 1998). This assumption corroborates the "rhizosphere effect" (Olson et al., 2003) described for terrestrial environments and confirms the plant promotion of microorganisms within salt marshes sediments. In winter, the plant activity normally decreases due to less sun hours and lower temperature, and a lower root exudation is expected (Palomino et al., 2005), which probably reduces the microbial growth in the rhizosphere. This could explain the encountered lack of differences ($P > 0.05$) between TCC in colonized and un-colonized sediments in this season.

In general, TCC did not vary significantly ($P > 0.05$) among plants rhizosediments, with exception of autumn, a season were TCC were higher in the *T. striata* rhizosediment. This probably could be explained by the *T. striata* phenological behavior, as the aboveground tissues of the plant die in autumn and fall down into the sediment (Laegdsgaard, 2006), being an additional source of nutrients to belowground microorganisms.

Several studies, focused on soil plants, have demonstrated that the presence of vegetation significantly enhanced the number of HD compared to un-vegetated soil (Corgié et al., 2003; Ho and Banks, 2006; Muratova et al., 2003). Information regarding the influence that rhizosphere of salt marsh plants might have on hydrocarbon degrading microorganisms is scarce, nevertheless, our study confirmed that salt marsh plants influence the abundance of such

communities. Indeed, the HD abundances in the rhizosediments of the exotic *T. striata* were always significantly ($P < 0.05$) higher than in un-colonized sediments, whereas in the invasive *P. australis* an increasing trend was also found.

Phenological behaviors (affected by the weather and climatic conditions), soil/sediment characteristics and salinity can also influence the success of microbial fostering by a particular plant, and therefore the contaminants remediation process (Hutchinson et al., 2003). In fact, higher ($P < 0.05$) HD counts were found in spring, encompassing the rise of temperature and the photoperiod. It is well known that temperature is especially linked to changes in soil microbial communities (Palomino et al., 2005). Microbial metabolism increases as temperature increases (Leahy and Colwell, 1990), and Coulon et al. (2005) demonstrated that HD abundance increased with the rise of temperature. Nevertheless, others conditions can be linked to the HD flourishing in this season such as oxygen levels, which are essential to aerobic HD, and are enhanced by the plant photosynthesis (Caffrey and Kemp, 1991). In addition, higher rates of root exudation, which increases with the photoperiod elongation, is an inevitable consequence of plant growth (vegetative and reproductive stages), and also likely to be affected by higher temperatures (Jones et al., 2004; Neumann and Römheld, 2000; Pramanik et al., 2000).

As discussed previously, *P. australis* and *T. striata* can successfully influence the movement, distribution and retention of hydrocarbons around their belowground tissues during high plant activity periods. A significant positive correlation between HD and TPH concentrations was seasonally found. But not all plant species have the same potential for fostering HD abundance. In fact, rhizosediments from *P. australis* and *T. striata* presented simultaneous higher HD and higher values of TPH concentrations than rhizosediment from *J. maritimus*. Plants can alter the microbial population, but these changes can be plant-specific (Kirk et al. 2005), associated to their root exudates (Palomino et al., 2005). In addition, this difference, between native and introduced plants, was likely due to the root morphology, as *P. australis* and *T. striata* have a fibrous root system, whereas *J. maritimus* has adventitious roots system borne in a horizontal rhizome. It is well known that fibrous and dense roots can penetrate a larger soil volume and their large rhizoplane surface area can be an

advantage (Aprill and Sims, 1990). On one hand, these roots have greater area of influence on the movement of compounds, and, on the other hand, they have a greater area for oxygen diffusion, stimulating aerobic HD. Oxygen diffusion in roots is determined by anatomical, morphological, and physiological characteristics (Colmer, 2003). In fact, plants with fibrous root systems have been proposed for phytoremediation of hydrocarbons in soils (Collins, 2007), and have been related to higher degradation of petroleum hydrocarbons (Merkl et al., 2005). These findings can assist our understanding on the mechanisms of plants to influence hydrocarbons remediation. However, a lack of knowledge regarding the role of the root system in hydrocarbons rhizoremediation is still evident.

Interestingly, the trend for TPH concentrations to decline during the seasons of higher plant activity was followed by an increase of HD, pointing to a period of higher rhizoremediation activity. Although the inputs and outputs of hydrocarbons were not controlled, because L3 sampling site was an open system, the mentioned dynamics was probably due to plant-microorganisms interactions. This report is one of the first attempts to describe the effect, "*in situ*", that different salt marsh plants might inflict on HD in a temperate estuarine environment, and therefore, with a wider geographical applicability. Despite the fact that no significant ($P > 0.05$) differences in terms of total microbial abundance were observed, the present study highlighted differences in the promotion of hydrocarbon degrading microorganisms among the different plants species, and also highlighted the vegetative and flowering stages as the periods of rhizoremediation activity potential. This is valuable information in order to predict the most adequate phase for rhizoremediation, which is less well characterized in the literature for salt marsh sediments. Indeed, the plant capabilities to foster microbial numbers in their rhizosphere, particularly HD, may be a useful tool to design cost-saving bioremediation strategies. A successful rhizoremediation strategy requires first and foremost plant species that stimulate specific pollutant-degrading biocenoses, whose microorganism can survive in sediments with a given climatic conditions and contamination levels.

4.5 Conclusion

This *in situ* study describes a one-year field-scale monitoring in a temperate estuarine area, and presents valuable outcome to the evaluation and management of the long-term viability of rhizoremediation technology with seasonal variation. The obtained results suggest that during the vegetative period (spring and summer), plants with fibrous and dense root system (*P. australis* and *T. striata*) tend to affect TPH distribution in a salt marsh by retaining hydrocarbons around their belowground tissues in a more efficient fashion than plants with adventitious root systems (*J. maritimus*). This study also confirmed the plant promotion of microorganisms within estuarine sediments, suggesting that distinct salt marsh plants can foster differently the development of hydrocarbon-degrading microbial population in its rhizosphere, particularly during the flowering season, when rhizoremediation activity is potentially higher. Moreover, root morphology can influence hydrocarbons degradation potential, having fibrous root plants a higher potential to foster hydrocarbon degraders, and therefore to remediate hydrocarbon pollution. Seasonality should also be taken into consideration when designing long-term rhizoremediation strategies.

Chapter 5

Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh

Published in Science of the Total Environment, 2013
Volume 458-460, 568-576

Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh

5.1 Introduction

Marine oil spills, particularly large-scale accidents, as the recent one in the Gulf of Mexico (Deepwater Horizon oil spill, 2010), may have catastrophic impact on coastal and estuarine environments. Although estuaries serve many ecological functions, they are ranked as the most anthropogenically degraded habitat types on Earth (Lytle and Lytle, 2001). Estuaries receive oil spills as a result of municipal and industrial wastewater discharges, urban runoff and oil leakage from boats and ships, as well as anthropogenic inputs from upstream catchments. Salt marshes, a common feature in temperate estuaries, are among the most productive ecosystems on Earth (Costanza et al., 1997), and extremely vulnerable to hydrocarbon contamination (Andrade et al., 2004). Oil pollution leads to the loss of biodiversity, destruction of breeding habitats of aquatic organisms and is hazard to the biota, including humans, being, the cleaning and recovery of those areas a difficult task (Zhu et al., 2004).

The use of plants and their associated microorganisms, an approach for the treatment of contaminated areas referred to as rhizodegradation or rhizoremediation (Gerhardt et al., 2009; Olson et al., 2003), can be considered less damaging and more cost effective than traditional cleanup methodologies. Plants can accelerate the bioremediation of soils and sediments contaminated with petroleum hydrocarbons (Barrutia et al., 2011; Davis et al., 2002; Lin and Mendelssohn, 1998; Xu et al., 2006; Zhang et al., 2010), with indigenous oil degraders providing less toxic, less mobile and/or less bioavailable products (Vidali, 2001).

Understanding the microbial ecology and its constraints on rhizoremediation of an oil contaminated site is extremely important, since the reliable use of microbes carrying degradative pathways often suffers from a lack of information on their diversity, survival and metabolic activity under different

environmental conditions (Galvão et al., 2005; Head et al., 2006; Lovley, 2003). Difficulties in studying microbial consortia in natural conditions arise because they are highly complex and the conventional cultivation methods can isolate only a small fraction of the microorganisms present in the environment (Malik et al., 2008). However, molecular techniques provide an opportunity, without the need for culturing, to investigate molecular composition and, therefore, increase our understanding of microbial diversity and functionality of the environment.

Contamination by oil is generally expected to reduce the biodiversity of the soil microbiota (Atlas et al., 1991), and eventually compromise their ecological functions. Heavy fractions of crude oil are known to persist for years in marsh sediments (*e.g.* Natter et al., 2012; Oudot and Chaillan, 2010; Vega et al., 2009). Despite the importance of salt marsh ecosystems, few studies (*e.g.* Bachoon et al., 2001) investigated the effects of oil pollution on the microbial community structure of salt marsh sediments, as well as the plant influence on the distribution and composition of hydrocarbon degrading microbial populations (Daane et al., 2001), which limits our ability to manage rhizoremediation strategies in the estuarine environment.

The aim of this study was to evaluate in microcosms the response of salt marsh microbial communities in un-colonized sediments and in sediments colonized by three distinct plants, to crude oil contamination. For that, microbial capacity to degrade hydrocarbons was assessed under different nutritional conditions. In addition, the effect of petroleum and nutrient amendments on the community structure and abundance was also assessed. Our hypothesis was that microbial communities from different sediments could respond differently to the petroleum contamination under different nutrient availability.

5.2 Materials and Methods

5.2.1 The study area

The study area, Lima River Estuary is the end member of an international watershed located in the NW of Portugal. The Lima River mouth has its right

bank modified by a city, Viana do Castelo, and has been subjected to several anthropogenic impacts.

For instance, in 2000, the bulk carrier 'Coral Bulker' ran aground at the entrance of the estuary, spilling 630 t of heavy fuel oil and 70 t of diesel oil, and severely affected the area (Moreira et al., 2004). Also, there is an important harbor, and a large shipyard leading to continuous petrochemical contamination through the activity ofcommercial and fishing vessels (Lima et al., 2007). Besides, in 2002 the oil tanker "Prestige" sunk off the coast of Galicia, NW Iberian Peninsula (42.15°N; 12.08°W (WGS84)), c.a. 280 km North-West of the Lima River Estuary, spilling 64,000 t of heavy fuel. Considering the extension of the area affected by the black tides (more than 1000 km), all estuaries on the coast of Galicia were affected by the oil spill (Andrade et al., 2004). The NW Atlantic coast of Portugal is also exposed to petrochemical contamination due to the presence of oil refining industry (located at Matosinhos) and two maritime harbors (located at Leixões and Viana do Castelo).

The Lima Estuary has a large salt marsh area, with a semidiurnal and mesotidal regime. The selected sampling site (L3, Figure 2.1) is located in the lower estuary. This site is colonized by assemblages of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata*, submitted to the same hydrologic conditions and salinity variations. These plants are perennial, belonging to different families: *J. maritimus* (native) belongs to the *Juncaceae* family, *P. australis* (invasive) belongs to the *Gramineae/Poaceae* family, and *T. striata* (exotic) belongs to the *Juncaginaceae* family (Coutinho, 1913; Sampaio, 1988; Franco and Afonso, 1994; 1998; 2003). *J. maritimus* and *P. australis* are common examples of ubiquitous species in the Portuguese salt marshes (Costa et al., 2009a), whereas *T. striata* is native from the Austral-Asian, South African and American territories, and its occurrence in Portugal is restricted to the north-western coast (Costa et al., 2009b). *J. maritimus*, the only plant with an appreciable rhizome structure, presented adventitious roots borne on the horizontal rhizome. As for *P. australis* and *T. striata*, both presented a fibrous and dense root system.

5.2.2 Sediment sampling and characterization

Sediment samples were collected in the summer (July) of 2010. Sub-surface sediments un-colonized and colonized (rhizosediments, sediment in contact with the plant roots) by *J. maritimus*, *P. australis* and *T. striata* were individually collected into sterile plastic bags. Each sediment was replicated three times for each plant assemblage, ca. 2 m from each other. All sediments were retrieved between 5 and 15 cm, the depth with the higher plant belowground biomass in the case of colonized sediments, being roots from rhizosediments removed as much as possible. Samples were transported to the laboratory in dark, refrigerated ice chests. Upon arrival, a portion of each sediment sample (initial sediments) was frozen at −20 ˚C for TPH analysis and DNA extraction. The remaining sediment was stored at 4 ˚C for the experiments and other analytical procedures, such as water and OM content and grain size. Further details on determination of water and OM content, as well as particle size distribution can be found in Ribeiro et al. (2011).

5.2.3 Batch incubation of rhizosediments for TPH degradation

It has been previously suggested (Aichberger et al., 2005) that shaking flasks was the faster (2–4 weeks), cheaper and less sample requiring test method to predict the hydrocarbon biodegradation potential, with a good indication of hydrocarbon degradability. As a result, this strategy was followed throughout the work. For the experiments, 10 ml (volume) of sediment samples (un-colonized and rhizosediments) was placed in 50 ml flasks, mixed with 20 ml of BH medium (supplemented with 2% sodium chloride to reflect in situ environmental salinity) and subjected to four different treatments: with and without additional nitrogen and/or petroleum amendment (Figure 5.1). The BH medium is a mineral-salt enrichment medium, suitable for the promotion of HD. Nitrogen is generally identified as the primary limiting nutrient for marsh vegetation (Crain, 2007). Although BH already has nitrogen in its composition, the amount may not be sufficient in face of the total carbon amount provided by the addition of oil (for the proportion C:N:P of 100:10:1). Therefore, to compensate a possible N-limitation, additional nitrogen was added (20 mM of NO_3^- as KNO_3 p.a., Merck) to the BH medium in two of the treatments. Arabian Light crude oil (supplied by an oil refinery), was shaken overnight in BH

medium prior to addition of 0.5 ml to flasks with sediments. The purpose of this process was to simulate the time frame between an offshore oil spill and the contact with salt marsh sediments, although under a real oil spill, the time will depend on wind, currents, and the distance to shore. Initial triplicate sediment samples with petroleum amendment were collected for analysis of TPH, and considered as T_0 samples. The remaining flasks, in triplicate for each treatment, were incubated at room temperature in the dark in an orbital shaker at 100 rpm. In a real oiled marsh, oxidized conditions are expected to prevail in rhizosediments, and anaerobic conditions to remain in un-colonized sediments. However, in order to standardize the conditions of experience, the experiment was conducted under oxidized conditions. The flasks were also manually shaken once every day to improve blending between oil and sediment. After 15 days of incubation, the sediment samples were removed and considered as T_{15} samples. For all sediment samples, total microbial abundance was estimated by the TCC, and HD abundance by the MPN protocol, as described below. A portion of each sediment sample (including T_0) was frozen at −20 ˚C until DNA extraction and TPH analysis to stop microbial activity.

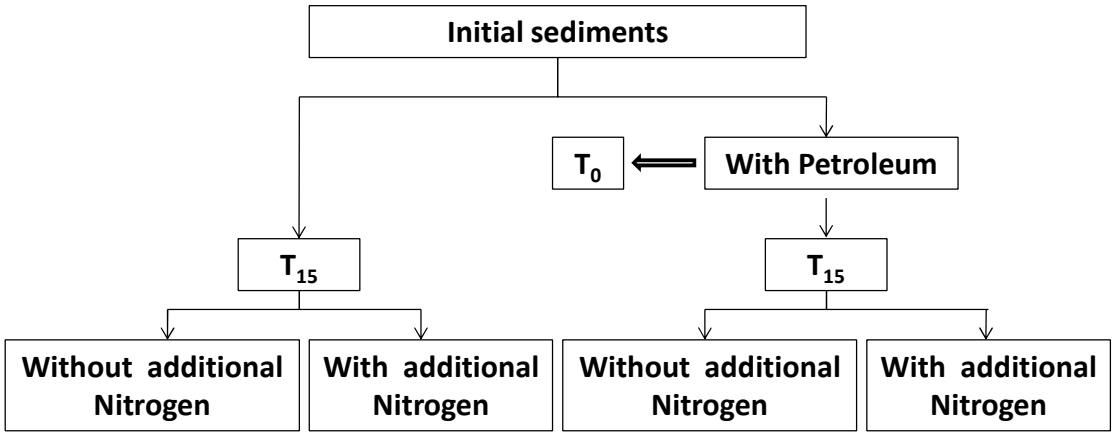

Figure 5.1 - Schematic representation of laboratory experiments. T_0 represents the beginning of the experiment; here samples with petroleum amendment were collected. T_{15} represents the end of the experiment, here samples, with and without petroleum amendment and/or additional nitrogen, were collected. Each treatment was carried out in triplicate.

5.2.4 Determination of TPH concentration by FTIR

Prior to TPH analysis, sediment samples were defrosted, dried at room temperature until constant weight and sieved through a nylon net of 2 mm mesh to remove large particles and roots. For TPH measurements, a previously optimized method was used (Couto et al., 2012). The total hydrocarbon content determination may include saturated hydrocarbons but also branched and n-CH_3 aromatics being the hydrocarbons normally more easily and quickly degraded. Briefly, about 1 g of sediment was mixed with anhydrous sodium sulfate (1:1 (w/w)), and tetrachloroethylene (≥99% spectrophotometric grade) (1:10 (w/v)) was added, being followed by an ultrasonic (Elma, Transsonic 460/H model) extraction for 30 min. The extracts were cleaned with deactivated silica gel (70–230 mesh), to remove non-mineral oil contaminants such as animal greases, vegetable oils, and other polar compounds, and refrigerated until analysis, usually within 1 h. The sample extracts were analyzed by FTIR (Jasco FT/IR-460 Plus) using a quartz cell of 40 mm path length (Infrasil I, Starna Scientific). Calibration standards (in tetrachloroethylene) were prepared using a stock standard solution of equal volumes of isooctane (≥99% ACS spectrophotometric grade), and hexadecane (99%) solutions. TPH were quantified by direct comparison with the calibration curve. Quality control tests were conducted by analyzing the certified reference material CRM350-100 (TPH in Sandy Loam Soil (C6–C35), from Resource Technology Corporation). The results were within the prediction interval of expected TPH concentration. Sample solutions spiked with known amount of hydrocarbons yielded recoveries between 82 and 135%. Results were expressed on a dry weight basis.

5.2.5 Microorganism abundance

5.2.5.1 Total cell counts

For total microbial abundance, TCC were obtained by DAPI direct count method (Porter and Feig, 1980; Kepner and Pratt, 1994), at 1.875× on an epifluorescence microscope (Labphot, Nikon, Japan). More details can be found in Ribeiro et al. (2011).

5.2.5.2 Hydrocarbon degrading microorganism counts

Abundance of HD was estimated using a modified MPN protocol (Haines et al., 1996; Wrenn and Venosa, 1996) in 96-well microtiter plates. Pre-filtered (0.2 µm) Arabian Light crude oil was the selective substrate for determination of total HD. Medium BH supplemented with 2% sodium chloride was used as the growth medium. Further details can be found in Ribeiro et al. (2011).

5.2.6 Bacterial community structure using automated rRNA intergenic spacer analysis (ARISA)

ARISA exploits the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes in the rrn operon (Ranjard et al., 2001). The IGS, which may encode tRNAs depending on the bacterial species, displays significant heterogeneity in both length and nucleotide sequence (Fisher and Triplett, 1999). For ARISA, DNA was extracted from 0.25 g wet weight of homogenized sediment samples (for each of the three replicates) using a modification of the CTAB (bromide-polyvinylpyrrolidone-b mercaptoethanol) extraction protocol described by Barrett et al. (2006). Quality of extracted DNA was evaluated by visualization on 1.5% agarose gels and each DNA preparation was quantified with the Qubit fluorometer (Invitrogen). For ARISA, extracted DNA was amplified using ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') primers set (Cardinale et al., 2004), which amplifies the ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson and Furhman, 2004). ITSReub was labeled with the phosphoramidite dye 6-FAM (6-carboxyfluorescein). PCRs were performed in duplicate 25 µl volumes containing between 2 and 6 ng of DNA, 400 nM of both primers, 200 µM dNTPs, 3× Taq PCR buffer, 2.5 U Taq DNA polymerase, 2.5 mM MgCl and 1 µg bovine serum albumin. The PCR mixture was held at 94 ˚C for 2 min, followed by 30 cycles of 94 ˚C for 45 s, 55 ˚C for 30 s, 72 ˚C for 2 min, and a final extension at 72 ˚C for 7 min. Duplicate PCR products were combined, visualized on 1.5% agarose gel, purified using a GFX PCR DNA purification kit (GE-Healthcare) and eluted in 30 ml of water. Purified product was quantified using the Quant-it dsDNA assay kit (Invitrogen), and a standardized amount of the purified PCR product was diluted 1 in 5 and mixed with 0.5 ml of ROX-

labeled genotyping internal size standard (ROX 1000, Applied Biosystems). The sample fragments were run on the ABI3730 XL genetic analyzer undertaken by STABVIDA Sequencing Facilities (Lisbon, Portugal).

5.2.7 Statistical and data analysis

The mean and standard deviation values for all parameters were calculated for each treatment, *i.e.* for each set of three flasks. Therefore, samples from each flask were independently analyzed. Initial sediment samples were run in triplicate, being mean and standard deviation values also calculated. Microbial enumeration data were log normalized prior to statistical analysis. Differences on HD, TCC, TPH and OM among salt marsh sediments were analyzed by a parametric one-way ANOVA. If any significant difference was detected, a multiple Tukey comparison test was performed to find out where the difference was. Correlation factors ($P < 0.05$) were analyzed by correlation matrices. All statistical tests were performed using the commercial software STATISTICA, version 7, StatSoft, Inc. (2004).

Operational taxonomic units (OTU) were analyzed by Peak Scanner™ version 1.0 Software (Applied Biosystems), and the data were transferred to a spread sheet for further processing. Fragments that differed by less or equal to 2 bp were considered identical, and fragments with Fluorescence Units below 200 were considered "background noise". Fragments less than 200 bp were removed since they were considered to be too short for intergenic spacer of bacteria. Then, values corresponding to peak areas were imported into the Primer 6 software package (version 6.1.11) (Clarke and Gorley, 2006). To study the bacterial community structure, data were normalized using the presence/ absence of pretreatment function, and samples were then analyzed using the Bray–Curtis similarity method and a MDS plot was then generated using the default parameters with a minimum stress of 0.01 to generate a configuration plot based on percent similarity. To assess the similarity of community composition of the bacterial assemblages obtained, an analysis of similarities (ANOSIM, based on Bray–Curtis similarity) was performed using the PRIMER software (Clarke and Gorley, 2006). The ANOSIM is a permutation-based hypothesis statistical test, an analog of the univariate ANOVA, which tests for differences among groups of multivariate samples from different locations or

experimental treatments. In our case, different treatments corresponded to petroleum and/or additional nitrogen.

Bacterial richness and diversity index values were calculated from the ARISA profiles to better address the ecological description of the bacterial community within samples. For these calculations, it was assumed that the number of peaks represented the species number (phylotype/genotype richness), and that the peak height represented the relative abundance of each bacterial species. The bacterial richness was expressed as the total number of unique OTU (peaks) identified in within each electropherogram. The Shannon–Wiener diversity index, which takes into account the number of species present and their relative importance within the assemblage, was calculated using the PRIMER software (Clarke and Gorley, 2006). Differences in bacterial diversity were tested using one-way ANOVA.

5.3 Results

5.3.1 Sediment characterization

In the initially samples, water; OM and Silt + Clay contents; and TPH concentrations in rhizosediments tended to be significantly ($P < 0.05$) higher than in un-colonized sediment (Table 5.1). Nevertheless, all sediments were characterized by small grain size particles, with more than 80% of total particle size being <0.25 mm, and high OM contents, presenting therefore similar characteristics. In addition, TPH concentrations were of the same order of magnitude in all sediment samples.

5.3.2 Hydrocarbon degradation potential after batch incubation

In control experiments without petroleum, TPH concentrations in sediments did not change significantly ($P > 0.05$) during the 15 d incubation, and kept roughly the same TPH concentrations (Table 5.1). The concentrations of TPH in sediment samples used in experiments with petroleum amendment for assessing TPH degradation rates are presented in Figure 5.2.

Table 5.1 - Water and organic matter (OM) contents (in percentage) (mean and standard deviation, n = 3), and particle size fractions (in percentage of dry weight) of collected un-colonized sediment and rhizosediments of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata*. Total petroleum hydrocarbons (TPH) concentrations (conc.) (mg g^{-1}, mean and standard deviation, n = 3) in these sediments are also included.

Sample	Water (%)	OM (%)	Silt + Clay (%)	Fine sand (%)	Medium sand (%)	Coarse sand (%)	Gravel (%)	TPH conc. (mg g^{-1})
Un-colonized sediment	47.7 ± 0.4	4.96 ± 0.09	55.4	27.7	16.7	0.1	*n.d*	0.26 ± 0.01
Rhizosediment of *J. maritimus*	55.3 ± 0.7	5.48 ± 0.02	59.8	32.8	7.5	*n.d*	*n.d*	0.34 ± 0.06
Rhizosediment of *P. australis*	62.2 ± 0.2	6.12 ± 0.09	61.8	31.0	7.1	0.1	*n.d*	0.62 ± 0.01
Rhizosediment of *T. striata*	52.7 ± 0.9	6.3 ± 0.2	67.7	30.2	2.1	0.1	*n.d*	0.45 ± 0.02

n.d - not detected

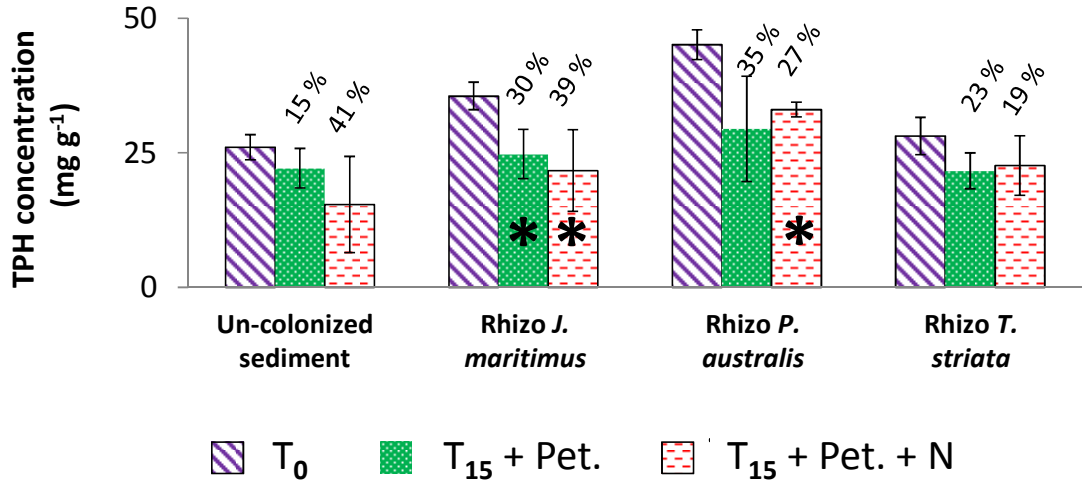

Figure 5.2 - Total petroleum hydrocarbon (TPH) concentrations (mg g^{-1}, mean and standard deviation, n = 3) in un-colonized sediments and rhizosediments (Rhizo) of *Juncus maritimus*, *Phragmites australis* and *Triglochin striata* used in experiments with petroleum amendment (Pet.). TPH concentration in the beginning of the experiment (T_0) and after 15 days (T_{15}) in sediments without and with additional nitrogen (N), and the respective TPH degradation percentages are shown (* shows the significantly different ($P < 0.05$) values when compared to those in T_0 samples).

The analysis of T_0 TPH showed differences among the studied sediments (un-colonized and colonized by the three plants), despite identical crude oil addition. These TPH concentrations were approximately 100 times higher than those initially present in the sediments. Nevertheless, there was a trend in all sediments for the decline of TPH concentrations after 15 days (T_{15} + Pet.), although, significant differences ($P < 0.05$) were only observed in *J. maritimus* and *P. australis* (with additional nitrogen, T_{15} + Pet. + N) rhizosediments. Additional nitrogen fostered a higher increase in the TPH degradation percentage in un-colonized sediment (T_{15} + Pet. + N), although, no significant ($P > 0.05$) differences were observed comparing these hydrocarbon degradation rates with the previous rates in sediments without amended nitrogen (T_{15} + Pet.).

5.3.3 Changes in microbial abundance and bacterial richness and diversity

Microbial abundance (TCC and HD) and bacterial richness and diversity are presented in Table 5.2. After 15 days of incubation, a general trend for TCC variation was observed. On the one hand, TCC in sediments without petroleum amendment (T_{15}) showed no significant ($P > 0.05$) changes, except in *P. australis* rhizosediment. On the other hand, TCC tended to increase significantly ($P < 0.05$) in sediments with petroleum amendment, where sediments with additional nitrogen (T_{15} + Pet. + N) showed a significant increase compared with sediments without additional nitrogen (T_{15} + Pet). Moreover, there was a trend for TCC in rhizosediments to be significantly ($P < 0.05$) higher than in un-colonized sediment, and no differences ($P > 0.05$) among rhizosediments of the distinct plants were recorded. As for TCC in the initial collected sediments, no significant differences ($P > 0.05$) were found among HD from rhizosediments. Also, HD counts of all three rhizosediments were significantly ($P < 0.05$) higher than in the surrounding un-colonized sediment. The effect of BH medium, with or without additional nitrogen on HD abundance by the end of the incubation, varied among sediments. Significant differences ($P < 0.05$) were observed between initial HD counts and T_{15} and T_{15} + N values, with the exception in *T. striata* rhizosediments. The highest stimulation of HD counts occurred in un-colonized sediments and the lowest in *T. striata* rhizosediments. The HD abundance in medium supplemented with

petroleum could not be quantified because of methodological saturation (all microtiter wells were positively scored, except the negative control).

Table 5.2 - Variation of microbial abundance estimated by total cell counts (log_{10} TCC g^{-1}, mean and standard deviation, n = 3); hydrocarbon degrading microorganisms estimated by most probable number (log_{10} HD g^{-1}, mean and standard deviation, n = 3); operational taxonomic units (OTU) richness and Shannon–Wiener diversity index (mean and standard deviation, n = 3) using ARISA results in un-colonized sediments and in *Juncus maritimus*, *Phragmites australis* and *Triglochin striata* rhizosediment (Rhizo) samples before (initial) and after the 15 day (T_{15}) experiment (with (Pet.) and without petroleum amendment and/or additional nitrogen (N)). The different superscript letters show the significant ($P < 0.05$) differences among treatments within each type of sediment.

Samples		Log_{10} TCC	Log_{10} HD	Statistics using ARISA results	
				OTU richness	Shannon-Wiener diversity
Un-colonized sediment	**initial**	8.76 ± 0.04^{A}	3.9 ± 0.2^{A}	286 ± 6^{A}	5.1 ± 0.1^{A}
	T_{15} (control)	8.70 ± 0.01^{A}	8.1 ± 0.5^{B}	196 ± 3^{B}	4.4 ± 0.1^{B}
	T_{15} + N	8.9 ± 0.1^{A}	9.3 ± 0.2^{C}	244 ± 4^{C}	4.7 ± 0.0^{B}
	T_{15} + Pet.	9.24 ± 0.05^{B}	m.s.	197 ± 4^{B}	3.8 ± 0.2^{C}
	T_{15} + Pet + N	9.35 ± 0.05^{B}	m.s.	184 ± 12^{B}	4.0 ± 0.1^{C}
Rhizo of *Juncus maritimus*	**initial**	9.14 ± 0.04^{A}	6.2 ± 0.4^{A}	248 ± 3^{AB}	5.0 ± 0.1^{A}
	T_{15} (control)	9.1 ± 0.1^{A}	11 ± 3^{B}	257 ± 1^{A}	4.9 ± 0.0^{A}
	T_{15} + N	9.16 ± 0.04^{A}	11 ± 2^{B}	243 ± 1^{B}	4.8 ± 0.0^{A}
	T_{15} + Pet.	9.27 ± 0.04^{B}	m.s.	242 ± 1^{B}	4.5 ± 0.1^{B}
	T_{15} + Pet. + N	9.79 ± 0.05^{C}	m.s.	200 ± 8^{C}	3.8 ± 0.1^{C}
Rhizo of *Phragmites australis*	**initial**	9.01 ± 0.01^{A}	6.1 ± 0.4^{A}	198 ± 6^{A}	4.4 ± 0.2^{AB}
	T_{15} (control)	9.23 ± 0.06^{B}	9.49 ± 0.01^{B}	261 ± 13^{B}	4.7 ± 0.2^{B}
	T_{15} + N	9.18 ± 0.06^{B}	11 ± 2^{B}	284 ± 5^{B}	4.9 ± 0.0^{B}
	T_{15} + Pet.	9.29 ± 0.06^{B}	m.s.	197 ± 5^{A}	4.0 ± 0.1^{A}
	T_{15} + Pet. + N	9.61 ± 0.04^{C}	m.s.	207 ± 10^{A}	4.0 ± 0.4^{A}
Rhizo of *Triglochin striata*	**initial**	9.00 ± 0.02^{A}	6.7 ± 0.3^{A}	225 ± 3^{A}	4.7 ± 0.1^{A}
	T_{15} (control)	9.2 ± 0.1^{B}	8 ± 3^{B}	211 ± 2^{B}	4.3 ± 0.1^{B}
	T_{15} + N	9.08 ± 0.09^{AB}	8 ± 1^{B}	231 ± 2^{A}	4.5 ± 0.1^{AB}
	T_{15} + Pet.	9.28 ± 0.08^{B}	m.s.	222 ± 2^{A}	4.4 ± 0.0^{B}
	T_{15} + Pet. + N	9.63 ± 0.07^{C}	m.s.	227 ± 7^{A}	4.4 ± 0.1^{B}

m.s.: methodological saturation

Bacterial richness and diversity measured in sediments by ARISA analyses were variable in sediment samples submitted to the treatments. In un-colonized sediments, initial sediment presented the higher richness and diversity. After

incubation and considering the control (T_{15}), the addition of petroleum treatment did not change ($P > 0.05$) richness, but the diversity declined significantly ($P < 0.05$).

For *J. maritimus* rhizosediments between the initial and the treatment without petroleum (T_{15} and T_{15} + N), no significant ($P > 0.05$) differences were generally observed in richness and diversity. The addition of petroleum reduced significantly ($P < 0.05$) the latter, but not the richness that declined significantly ($P < 0.05$) only with the addition of nitrogen. *P. australis* rhizosediments contaminated with petroleum (T_{15} + Pet.) did not present significant ($P > 0.05$) differences in richness and diversity compared with the initial rhizosediment. Nevertheless, richness and diversity in control sediments (T_{15}) were significantly ($P < 0.05$) higher than in rhizosediments contaminated with petroleum. Finally, *T. striata* rhizosediments, generally, maintained richness and diversity, with no significant ($P > 0.05$) differences among sediments of the different treatments. Therefore, with exception of *T. striata* rhizosediments, when compared with control (T_{15}), the petroleum treatment reduced significantly ($P < 0.05$) the overall bacterial diversity.

As a general rule, initial sediments presented significantly ($P < 0.05$) higher Shannon indices, contrasting with sediments contaminated with petroleum and with additional nitrogen (T_{15} + Pet. + N). As expected, the results found in total microbial abundance presented the inverse trend. In fact, a significant negative correlation was obtained considering TCC and the diversity ($r = -0.59$; $P < 0.05$; $n = 20$).

5.3.2 Changes in the bacterial community structure

ARISA analysis was performed in the initially collected sediments and in three replicates from each treatment at the end of the experiment to evaluate shifts in the bacterial community structure related to the plant presence, plant species, petroleum contamination and/or nutritional conditions (Figures 5.3 and 5.4). The most relevant information was given by the distribution of the OTU (bacteria phylotypes) in the different samples, since it corresponded to differences in their genetic structure. Despite the biological variability among samples and the bias that might be introduced mainly by DNA extraction and PCR amplification, ARISA electropherograms of replicates were highly similar.

Replicates were grouped together at 95% similarity, suggesting good experimental replication.

The MDS plot (Figure 5.3) indicated that the most dissimilarity was observed between bacterial community of un-colonized sediment and rhizosediments, which were clustered together at 35% of similarity. Additionally, at 50% of similarity, a clear separation among rhizosediment bacterial community structure was observed, with significant (ANOSIM, $P < 0.05$) differences, clustering independently each of the three rhizosediments. This indicates that the presence of plants and the plant species may act as a selection factor for the bacterial assemblage composition.

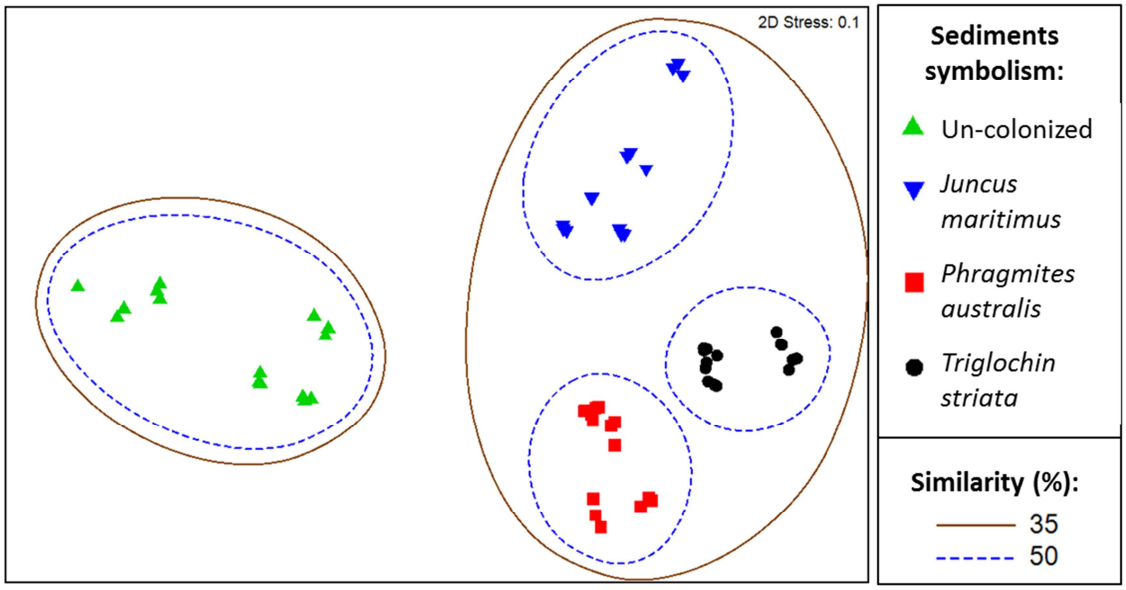

Figure 5.3 - Multidimensional scaling (MDS) ordination based on Bray–Curtis similarities on the presence/absence matrix obtained from ARISA fingerprints of bacterial communities from un-colonized sediments and from *Juncus maritimus*, *Phragmites australis* and *Triglochin striata* rhizosediment samples before and after the experiment (with and without petroleum amendment and/or additional nitrogen, all sediment replicates are included). Samples enclosed by circle clusters at 35% and 50% similarity.

For each type of sediment, important differences among treatments were observed in terms of bacterial community structures (Figure 5.4). After 15 days of incubation, sediment without petroleum amendment (T_{15} and T_{15} + N) was more similar to the initial sediment than to the treatments with petroleum (T_{15}

+ Pet. and T_{15} + Pet. + N), showing that the structure modifications induced by the experimental procedures were less discriminatory than the petroleum amendment. Moreover, dissimilarity between treatments with and without petroleum clearly indicated that, after the plant species-specific bacterial communities, petroleum amendment was the factor that induced the major changes in the bacterial community profiles, being followed by the nutritional conditions.

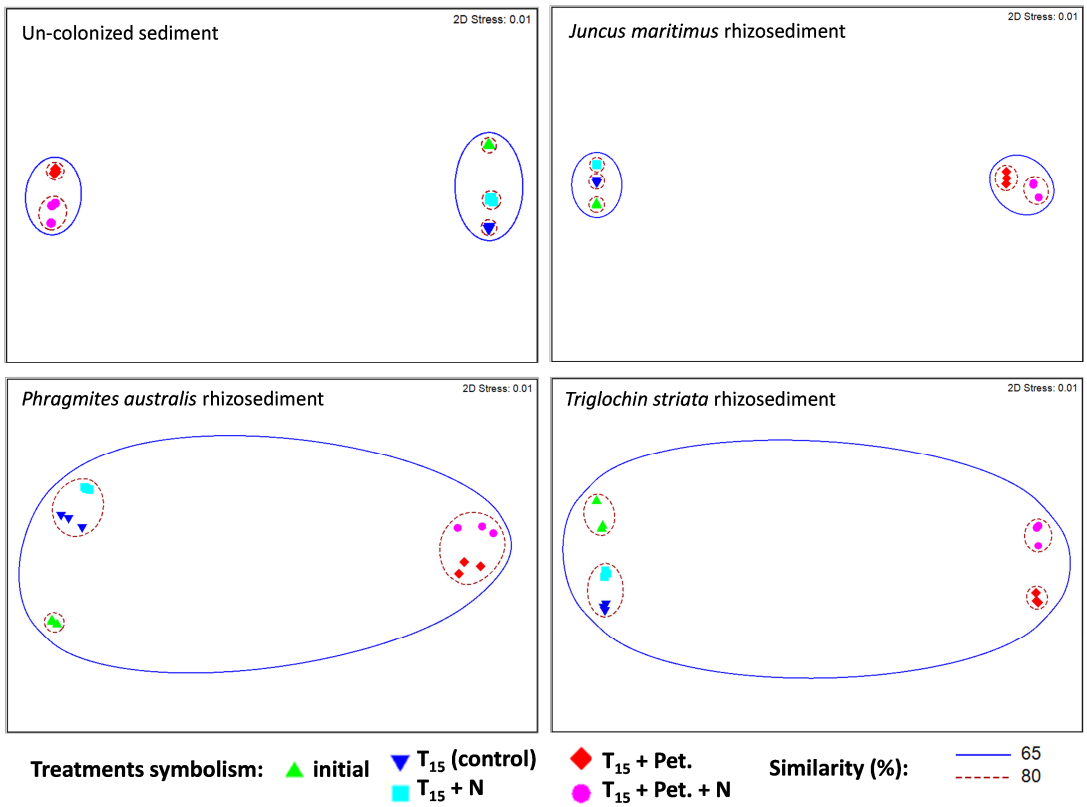

Figure 5.4 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities on the presence/absence matrix obtained from ARISA fingerprints of bacterial communities from un-colonized sediments and from *Juncus maritimus*, *Phragmites australis* and *Triglochin striata* rhizosediments at the beginning (initial), and after the experiment (T_{15}), with petroleum (Pet.) amendment and/or additional nitrogen (N) (all sediment replicates are included). Samples enclosed by circle clusters at 65% and 80% similarity.

The MDS plots (Figure 5.4) for each of the four sediments showed a trend for a bacterial assemblage cleavage on the bacterial community structure, with

significant (ANOSIM, $P < 0.05$) differences among treatments. Moreover, un-colonized sediments and *J. maritimus* rhizosediments at the end of the experiment showedmore dissimilarities, at 65% of similarity, being the sediments separated in treatments with and without petroleum. However, bacterial community structure from *P. australis* and *T. striata* rhizosediments was more similar, being the rhizosediments with different treatments all clustered, at 65% of similarity.

5.4 Discussion

A widely accepted hypothesis is that microorganisms are ubiquitous, but under strong environmental selection (Böer et al., 2009). Several studies (Blum et al., 2004; Cordova-Kreylos et al., 2006; Franklin et al., 2002) have evidenced that microbial community composition in salt marsh sediments was determined by the spatial variability, dominant plant, natural sediment characteristics and even the type of pollutants. In the case of an oil spill in these sensitive ecosystems, little is known in what way microbial communities respond to petroleum contamination. Aiming this purpose, we have evaluated, under laboratory controlled conditions, if petroleum contamination can foster changes in bacterial diversity and community structure of different salt marsh sediments as well as the capacity of these communities to degrade hydrocarbons.

A relatively short time of experiments was selected (15 days). The overall microbial community response was probably not completed after 15 days. The heavy fraction of oil may persist for months or even years in the environment (*e.g.* Natter et al., 2012). Nevertheless, a clear response in terms of biodegradation of some hydrocarbon fraction, namely the lighter ones, within this time frame was expected. Other studies (*e.g.* Head et al., 2006; Païssé et al., 2010) hypothesized that rapid and extensive change in hydrocarbonoclastic populations is established within 2 weeks. These changes are probably related with the removal of alkane hydrocarbons, which are generally accepted as the first to be biodegraded.

Our results clearly indicate that the presence/absence of plants and the plant species were the two most important factors for determining bacterial

community structure, being followed by other factors (petroleum amendment and nutritional conditions). It is well known that plants exert an important influence and can shape the structure and composition of microbial communities by enhancing their activity by root exudate composition and quantity (Bais et al., 2006; Koranda et al., 2011; Marschner et al., 2004). Several studies (*e.g.* Aira et al., 2010; Appuhn and Joergensen, 2006; Bais et al., 2006) also revealed that root exudates, which largely determine the composition of the rhizosphere microbial community, depend, among other factors, on plant species.

In this study, the multivariate analysis of ARISA profiles indicated that, within each sediment type, the presence of petroleum caused an appreciable shift in the bacterial community structure. These findings were consistent with coastal sediment studies (Païssé et al., 2010), that also highlighted the change in the structure of the bacterial community after 15 days of exposure to crude oil addition. Moreover, the amendment of additional nitrogen further shifted the microbial community structure within sub-clusters formed by sediments with and without petroleum, highlighting that nutritional conditions can indeed alter microbial communities. This nitrogen supplementation in the form of KNO_3 could stimulate denitrifying populations, inducing changes in the microbial community structure, at least transiently, as observed in other studies (Spain et al., 2007; Vázquez et al., 2009).

Since one of the main purposes of the study was to assess the response of salt marsh microbial communities to a petroleum contamination, the bacterial richness and diversity were also calculated to characterize microbial assemblages. Several microbial studies (*e.g.* Hewson and Furhman, 2004) have used diversity indices and estimated species richness from ARISA analyses. It is well known that all PCR-based fingerprint methods suffer from the same sources of bias, linked for example to the DNA extraction and the generation of artifactual heteroduplex fragments during PCR amplification (*e.g.* Acinas et al., 2005). In spite of the criticisms of some authors concerning the use of ARISA to assess bacterial richness or diversity (*e.g.* Bent et al., 2007), for comparative purposes, linking levels in habitats or the impact of different treatments on the diversity, ARISA could be very useful, and have been used in several studies (*e.g.* Hewson and Furhman, 2004; Or and Gophna, 2011). These tools can provide a considerable amount of information about the species

composition, and allows comparisons among estimates of microbial diversity (Hughes et al., 2001). ARISA profiles reflect the composition of the microbial community, including non-cultivable ones, and allow us to extend our understanding and to evaluate the changes in microbial communities. Due to the experiment design, anaerobic bacteria, particularly those from un-colonized sediments, might not have been potentiated. Nevertheless, when comparing the initial sediments with the control at the end of the experiment, we realized that experimental conditions were less discriminant than the sediment type or the hydrocarbon addition.

In our study, with exception of *T. striata* rhizosediments, richness and diversity decreased in the petroleum-contaminated samples compared with sediments without petroleum amendment, particularly in samples with additional nitrogen. We found a negative correlation between TCC and diversity. In fact, petroleum contamination led to TCC increase, which coincided with a diversity decrease, with the exception of *T. striata* rhizosediments. The OTU richness decrease suggests a deleterious effect of added hydrocarbon in the most sensitive species, as previous reported by Grötzschel et al. (2002). Nevertheless, we hypothesize that the main effect of petroleum amendment and nitrogen amendment was the stimulation of HD and denitrifying bacteria, an outcome presented in several studies (*e.g.* Yakimov et al., 2005). The stimulation increased the relative importance of these bacteria within the assemblage, which led to the diversity reduction. Curiously, petroleum amendment in *T. striata* rhizosediments showed no effect on microbial richness and diversity. Also, it was observed that this rhizosediment, together with the one from *P. australis*, presented the higher similarities (above 65%) between treatments with and without petroleum, pointing to an apparent resistance, being the resistance the degree to which microbial composition remains unchanged in the face of a disturbance (Allison and Martiny, 2008). In fact, these microbial communities seem to be able to resist to petroleum contamination better than those from *J. maritimus* rhizosediment or un-colonized sediments. This microbial community resistance could be related with the specificity of the associated plant root system, since *T. striata* and *P. australis* present a fibrous and dense root system, while *J. maritimus* have an adventitious root system. Moreover an *in situ* characterization carried out by the authors (Ribeiro et al., 2013a),

highlighted the capabilities of these plants to foster HD in rhizosediments throughout a phenological cycle. Indeed, initial rhizosediments from these plants presented the highest TPH concentrations, therefore, we hypothesize that the microbial community was familiarized with hydrocarbons, and not greatly impacted with further petroleum addition. Nevertheless, additional studies are needed to understand the significance of microbial community shifts associated with diversity decrease on ecological function. In fact, microbial ecology still lacks a strong predictive framework to interpret the functional consequences of changes in microbial composition due to petroleum contamination. Moreover, the study of resilience, *i.e.* the rate at which microbial composition returns to its original composition after being disturbed (Allison and Martiny, 2008), could be a useful and sensitive way of monitoring the impact and recovery of petroleum-contaminated sediments.

As mentioned, petroleum amendments fostered, in general, the abundance of the microbial communities, which was consistent with other studies (Bachoon et al., 2001; Röling et al., 2002; Yakimov et al., 2005). The fact that TCC and HD in rhizosediments were, in general, significantly ($P < 0.05$) higher than in un-colonized sediment, but without differences among those rhizosediments could be explained by the "rhizosphere effect" in salt marshes, recently reported by the authors (Ribeiro et al., 2011). Thus, the increased HD abundance in the presence of petroleum could be explained by the readily available petroleum derived carbon used by heterotrophic organisms as carbon source. In fact, Pearson et al. (2008) showed that by the end of a 15-d experiment, up to 26% of bacterial biomass was derived from consumption of the freshly spilled oil. This could explain the slight TCC increase observed in our study in sediments without additional nitrogen amendment. In addition, the amendment with nitrogen increased the total microbial abundance, pointing to the importance of nitrogen limitation for biomass production of microbial communities in sediments contaminated with petroleum. It is well known that lower levels of nitrogen limit microbial growth (Harayama et al., 1999). Unfortunately, it was not possible to access the influence of nitrogen amendment on the HD due to the above mentioned methodological saturation. Nevertheless, we assumed that the increase in TCC was due to HD development. The addition of nitrogen has been repeatedly reported (*e.g.* Zhu et al., 2004), as an effective strategy to promote microbial growth and to boost

hydrocarbon degradation in sediments contaminated with petroleum. Our results concur with this, although hydrocarbon degradation rates, particularly in rhizosediments, did not respond to the additional nitrogen. Firstly, in sediments contaminated with petroleum, but without nitrogen amendment, a decrease in TPH concentration after 15 days was observed, with microorganisms degrading 15–35% of the initially present petroleum hydrocarbons. It should be noted that shaking microcosms to improve blending between oil and sediments can result in higher rates of biorespiration, and consequently higher hydrocarbon degradation rates than in a real estuarine environment, particularly in what un-colonized sediments are concerned. Nevertheless, hydrocarbon degradation rates were higher in rhizosediments than in un-colonized sediment, which could be explained by a nutrient richer environment due to the above mentioned "rhizosphere effect". Plants can provide nutrients by root exudation, which stimulate microbial activity (Kuiper et al., 2004). With the addition of nitrogen to sediments contaminated with petroleum, degradation rates increased, with microorganisms degrading 19–41% of the initially present petroleum hydrocarbons. The higher degradation rate occurred in un-colonized sediment, which points to an initially nutrient-poorer environment, while degradation rates in rhizosediments were only slightly affected by the additional nitrogen. The less effect of additional nitrogen on hydrocarbon degradation rates might be due to nutrient advantage in those rhizosediments, which leads already to a maximum extent of hydrocarbon degradation without nitrogen addition during the 15 days.

In sediments without petroleum addition, differences in TPH concentration were not statistically ($P > 0.05$) significant after 15 days. This indicates that hydrocarbons initially present in sediments were, probably, not available. The sediments used in this study, despite some small differences, presented high contents of Silt + Clay and OM (Table 5.1), characteristics that may influence negatively hydrocarbon remediation. Small grain particles and high organic content are thought to be the main factors that limit hydrocarbon bioavailability, which reduces the rate and extent of degradation (Ribeiro et al., 2012). In fact, by altering sediment properties, like the organic carbon and small size particle fractions, plants can also affect the distribution and concentration of hydrocarbons by retaining them around their belowground

tissues. Indeed, TPH concentrations (Table 5.1 and Figure 5.2) showed differences among sediments colonized by distinct plants, which reflected the sediment capacities to retain hydrocarbons, eventually due to the OM content (Ribeiro et al., 2011).

The obtained results indicated that the plants could influence the structure of the bacterial communities and foster the number of hydrocarbon degraders and the response of the microbial community to an oil spill and the subsequent degradation of the pollutant. One should take into consideration that experiments were carried out under laboratory-controlled conditions, isolating therefore the microbial activity from physical-chemical factors. Microcosm experiments could result in a precise evaluation of the potential impact of contaminants on soil microorganisms, as several erratic variations are minimized (Ranjard et al., 2006). This strategy was followed in this work; nevertheless, information gathered on the hydrocarbon degradation potential should be validated under conditions more similar to those occurring in a real estuarine environment.

5.5 Conclusion

Our results clearly suggest that distinct salt marsh microbial communities responded in the same way to the petroleum contamination, *i.e.* increasing microbial abundance, changing microbial community structure and decreasing microbial diversity. Nevertheless, sediment type (associated to plant species) seems to be the most important factor for determining rhizosphere community structure, being followed by the petroleum amendment. In fact, petroleum contamination of different salt marsh sediments did not lead to increasing similarity of the different microbial communities. Petroleum contamination and the amended nitrogen seemed to induce a quantitative (abundance increase) and qualitative shift (diversity decrease) on salt marsh microbial communities. These communities, particularly those associated with *J. maritimus* and *P. australis* roots, displayed a potential to degrade petroleum hydrocarbons, with TPH degradation percentage between 15% and 41%. The degradation process was, probably, dependent on the nutrient demands of the tested sediments. These nutritional requirements should be taken into account when developing bioremediation strategies in estuaries.

Chapter 6

Potential of salt marsh plants for the removal of petroleum hydrocarbon: rhizoremediation, biostimulation or bioaugmentation?

Submitted to Journal of Hazardous Materials

Potential of salt marsh plants for the removal of petroleum hydrocarbon: rhizoremediation, biostimulation or bioaugmentation?

6.1 Introduction

Salt marshes provide an ecologically important environment and numerous benefits for humans (Boorman, 1999). These important ecosystem services are often at risk when oil spills occur due to accidental discharges, leakage from boats and storage tanks, municipal and industrial wastewater discharges, and urban runoff. The need to restore sensitive petroleum hydrocarbon contaminated sites requires the application of adapted strategies that cause the minimum environmental and ecological impact.

Bioremediation, the acceleration of pollutants removal by organisms, *e.g.* microbial communities, is commonly used as "*in situ*" environmental friendly cleanup method. There are two basic forms of bioremediation in the case of microorganisms: biostimulation (BS), *i.e.* the injection of nutrients to induce microbial propagation of the native microbial population; and/or bioaugmentation (BA), the addition of enriched microbial cultures, resistant to the pollutant, to enhance its degradation (Tyagi et al., 2011). Another cost-effective option for the treatment of contaminated areas is the use of plants, particularly their roots and associated microorganisms, in a process referred to as rhizoremediation (RR) (Gerhardt et al., 2009).

Several studies (*e.g.* Lin and Mendelssohn, 1998; Maqbool et al., 2012) showed accelerated hydrocarbon degradation in the rhizosphere region compared to un-colonized soils/sediments. Salt marsh plants tolerate salinity, which make them potentially suitable for hydrocarbons remediation in coastal environments, where accidental oil spills hazard is potentially high. So, enhancing salt marsh vegetation in oil affected sediments could be a useful technique for the reestablishment of coastal wetlands. Nevertheless, data on temperate salt marsh plants that may reduce hydrocarbon levels in contaminated sediments are still lacking, being that information essential for a

successful restoration and remediation of oil-impacted habitats. Some laboratory and field trials (reviewed in Zhu et al., 2004) focused mainly on the influence of temperature, nutrients, oxygen, pH and salinity on microbial degradation of hydrocarbons in salt marshes. However, only very few studies compared microbial hydrocarbon degradation in un-colonized and colonized sediments, and the influence of supplementary bioremediation approaches (*e.g.* Garcia-Blanco et al., 2007; Wright and Weaver, 2004; Wright et al., 1997). The efficacy of bioremediation strategies in a holistic and realistic approach have been reported as unsuccessful in salt marshes contaminated with crude oil (*e.g.* Tate et al., 2012). Oxygen availability may be often the limiting factor for oil biodegradation in estuarine wetlands, and no feasible techniques are currently available for increasing oxygen availability in such environments (*e.g.* Zhu et al., 2004).

Therefore, the understanding of how plants, nutrient and hydrocarbon degrading microorganisms addition, or even their combination, may potentiate oil biodegradation in salt marshes remains an unsolved challenge. Research on rhizoremediation of hydrocarbons contaminated salt marsh sediments is broadly implemented in north America (Zhu et al., 2004; Wright et al., 1997; Wright and Weaver, 2004; Garcia-Blanco et al., 2007; Tate et al., 2012), however studies addressing temperate salt marsh areas in the European Atlantic coast are scarce (*e.g.* Vega et al., 2009). Moreover, studies in north America have focused on a handful of native plants, particularly *Spartina alterniflora*. Salt marsh plant species around the world can be different (Adam, 2002), but also variable in terms of root morphology, root exudation, root decomposition, and associated microbial communities (Lee et al., 2008). Therefore, different plant species and/or different combinations of bioremediation treatments should be tested to identify and overcome factors influencing rhizoremediation efficacy.

The overall aim of this study was to assess the suitability of two salt marsh plants, *Juncus maritimus*, and *Phragmites australis* commonly found in temperate estuaries, for petroleum hydrocarbons rhizoremediation, by means of a 5-month greenhouse experiment in order to evaluate the efficiency of different bioremediation strategies.

6.2 Materials and Methods

6.2.1 Sampling

Sediments and plants, *J. maritimus* and *P. australis*, were collected in April of 2012 at low tide from a salt marsh located in the Lima River Estuary. *J. maritimus* presented an appreciable rhizome structure, with adventitious roots borne on the horizontal rhizome, and *P. australis* presented a fibrous and dense root system. Further details about these plants and sampling location can be found elsewhere (Ribeiro et al., 2013a).

Only green plants without a senescent appearance and with similar size were collected together with the colonized sediment (rhizosediment) attached to their roots (cubes of c.a. 15 cm). Simultaneously, un-colonized sediment, located within 2 m of the colonized sediment, was collected between 5 and 15 cm, the depth with the higher plant belowground biomass in the case of rhizosediments. After collection, samples were immediately carried into the laboratory. A portion of each sediment samples was frozen at -20 ºC for TPH background analysis, and another portion was stored at 4 ºC for additional procedures, such as water and OM content, grain size and microbiological procedures (carried out within 24 h) according to Ribeiro et al. (2011).

6.2.2 Preparation of sediments, plants and microbial consortia

In the laboratory, sediments were carefully separated from roots as much as possible. Plants were kept in plastic vessels, containing one quarter-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950), for 3 days until transplantation.

Sediments were stored in separate plastic vessels (20 cm x 50 cm x 120 cm) according to their kind (un-colonized sediment, *J. maritimus* and *P. australis* rhizosediments) to maintain their indigenous microbial communities. Sediments were manually homogenized (loose plant rhizomes and roots were removed) divided into 2.5 L portions of wet sediment and transferred to individual aluminum foil containers. Then, sediments were mixed manually with Arabian Light crude oil (supplied by an oil refinery) to a concentration of 5

ml $L^{-1}_{wet\ sediment}$. Also, 0.5 L of dechlorinated (through charcoal filter) tap water was added to facilitate sediment homogenization. Sediments remained for 48 hours under a hood to allow for partial aging of the contamination and adsorption of crude oil to the sediment particles. A batch of uncontaminated sediments was also prepared using the same procedure, except the crude oil addition. All batches were left for 48-h at room temperature until further processing.

For the preparation of the microbial consortium to be used in BA treatments, 100 ml of homogenized sediment (in triplicate for each sediment type) were mixed with 200 ml of BH broth (Sigma for microbiology CAS). The medium is usually used for growing hydrocarbon degrading microorganisms, supplemented with 2 % sodium chloride (NaCl p.a., Merck), and with 20 mM nitrogen (as KNO_3 p.a., Merck). Pre-filtered (0.2 µm) crude oil was used as selective substrate to enrich indigenous microorganisms potentially capable of degrading hydrocarbon compounds. The flasks with the inoculum were incubated at room temperature in the dark in an orbital shaker at 100 rpm for five days. At the fifth day flasks were manually shaken, left stand for 1 min, and each replicate was transferred to growth vases (see below).

6.2.3 Experimental setup

The experiment was conducted in a greenhouse exposed to natural light and environmental temperature conditions. Thirty-six plastic vases (5 L each) were fitted with plastic spigots, lined inside with aluminum foil. A layer of pebbles (0.5 L) was placed on the bottom to allow water circulation, minimize sediment loss, and prevent clogging. Then, vases were filled with sediments from the previous section. A small portion of sediment of each vase was collected and frozen at -20 ºC until hydrocarbons analysis, and considered as time zero (T_0) samples (Figure 6.1).

In vases with rhizosediments, ca. 20 and 50 culms (shoots with intact roots, washed of sediment) of *P. australis* and *J. maritimus*, respectively, were transplanted into their respective rhizosediment. Addicionally, two treatments (BS and BA) were applied to crude oil contaminated sediments, either colonized or un-colonized (Figure 6.1). Each treatment was replicated three times.

During the experiment, plants and sediments were watered with a solution supplied by an automated irrigation system, regulated to mimic natural tide dynamics. Vases were, therefore, under a semidiurnal tidal cycle effect. The irrigation solution consisted of a low nutrient saline solution (1/400 strength Hoagland nutrient solution and 1 % NaCl). The N and P concentrations in the irrigation solution were identical to those in the water flooding the location where the plants were collected. This methodology was applied to all thirty-six vases, and consisted in the only action applied to the control, natural attenuation (NA) and RR vases.

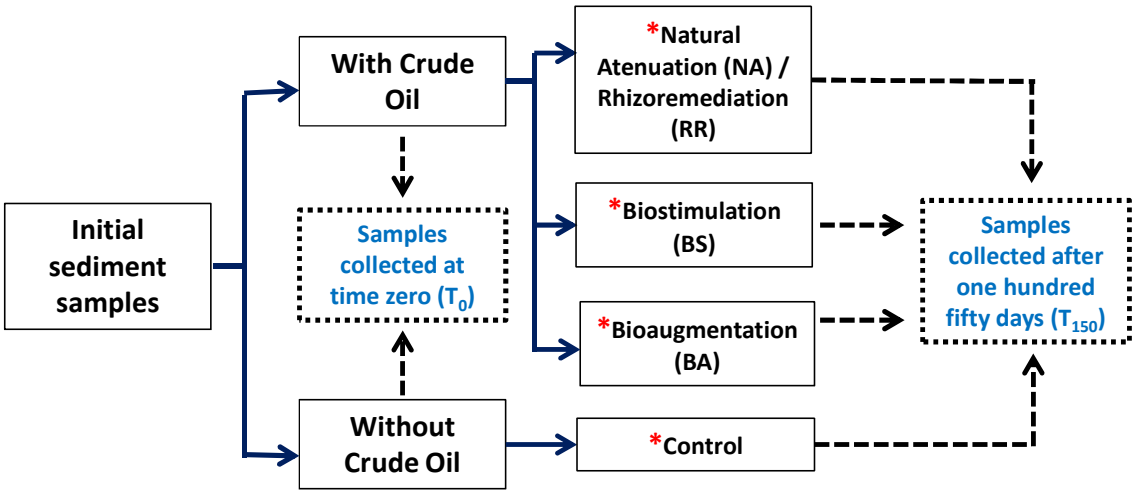

Figure 6.1 - Experimental design of the work. Un-colonized sediments and *Juncus maritimus* and *Phragmites australis* rhizosediments samples were collected at the beginning (T_0) and after the 150 days (T_{150}) experiment in untreated and crude oil spiked sediments. Distinct treatments (*) were applied to 3 replicates of un-colonized sediment + 3 replicates of sediment colonized by *J. maritimus* + 3 replicates of colonized sediment by *P. australis*.

At the 2nd day, BA treated sediments were inoculated with 100 mL of the respective microbial inoculum previously prepared. For both BS and BA treatments, sediments were additionally watered once a week with 0.5 L of a 1/4 Hoagland nutrient solution to increase the load of nutrients to the system.

After 5 months, samples from sediments in contact with the root system were collected, homogenized and portioned to aluminum foil and sterile plastic bags, and considered as time one hundred fifty days (T_{150}) samples. Samples

were preserved in two different ways: a portion was frozen at -20 ºC until hydrocarbon analysis, and another portion was refrigerated at 4 ºC for microbial analysis. Plants, *J. maritimus* and *P. australis*, were thoroughly rinsed with deionized water and divided into above and belowground parts for biomass measurements.

6.2.4 Determination of TPH concentrations by FTIR

Prior to hydrocarbons analysis, sediment samples were defrosted, dried at room temperature until constant weight and sieved through a nylon net of 2 mm mesh to remove large particles and loose roots. For TPH measurements, a previously optimized method was used (Couto et al., 2012). Hydrocarbons were extracted with tetrachloroethylene (≥ 99% Spectrophotometric grade, Sigma Aldrich) in an ultrasonic bath (Elma, Transsonic 460/ H model) at room temperature for 30 min, being the extracts analyzed by FTIR. Quality control tests were conducted by analyzing the certified reference material CRM350-100, with TPH concentration within the prediction interval. Sample solutions spiked with known amount of hydrocarbons, yielded recoveries between 82 and 135 %.

6.2.5 Gas chromatography-flame ionization detection (GC-FID) determination

To have a chemical profile of *n*-alkanes hydrocarbons (a smaller range than the one covered by FTIR determination), analyses by gas chromatography with flame ionization detection (GC-FID) of dry sediments were also carried out. Sediments were firstly extracted with a mixture of n-hexane (95 %, UV-IR-HPLC, PAI-ACS, Panreac) and acetone (≥99.9 %, CHROMASOLV® plus, HPLC, Sigma-Aldrich) (1:1 (v/v)) in the above mentioned ultrasonic conditions. The extracts were cleaned with Florisil® (60-100 mesh, Fluka) and analyzed in a GC-FID (Varian 3800) with a column 30 m×0.25 mm×0.25 µm (Varian Factor Four, VF-5ht), following the conditions described in Saari et al. (2007). The hydrocarbon range window measured was between nonane (C_9) and tetracontane (C_{40}). Composite samples were analyzed for each treatment.

6.2.6 Hydrocarbon degrading microorganisms counts

Abundance of HD was estimated using a modified MPN protocol (Haines et al., 1996; Wrenn and Venosa, 1996) in 96-well microtiter plates. Pre-filtered (0.2 µm) Arabian Light crude oil was the selective substrate for determination of total HD. Medium BH supplemented with 2% NaCl was used as the growth medium according to Ribeiro et al. (2011).

6.2.7 Statistical and data analyses

The mean and standard deviation values were calculated for each treatment, ie, for each set of three vases. For that, samples from each vase were independently analyzed. Initial sediment samples were analyzed in triplicate for all relevant parameters, being mean and standard deviations values also calculated. Microbial enumeration data were normalized by logarithm (log_{10}) transformation prior to statistical analysis. Significant differences ($P < 0.05$) between T_0 and T_{150} TPH concentrations were evaluated using t tests. Root biomass, HD, water contents, OM and Silt + Clay particle size among sediments were analyzed by a parametric one-way ANOVA. If a significant difference was detected, a multiple Tukey comparison test was performed to find out where the difference was. All statistical tests were performed using the commercial software STATISTICA, version 7, StatSoft, Inc. (2004).

6.3 Results

6.3.1 Sediments characterization

Sediments were characterized in terms of water and OM content as well as grain size distribution (Table 6.1). All sediments, independently of the presence of plants, were dominated by small grain size particles, with over 80% of total particle size being <0.25 mm (Silt + Clay + fine sand). In general, rhizosediments showed higher water and OM contents than un-colonized sediments.

Table 6.1 - Water and organic matter (OM) contents (in percentage), and particle size fractions (in percentage of dry weight) (mean and standard deviation, n = 3) of un-colonized sediment and rhizosediments of *Juncus maritimus* and *Phragmites australis* used in the experiments.

	% Water	% OM	Particle size fraction (% relatively to total weight)				
			Silt + Clay	Fine sand	Medium sand	Coarse sand	Gravel
Un-colonized sediment	52 ± 1	4.9 ± 0.2	57 ± 2	28.3 ± 0.6	14 ± 2	0.1 ± 0.1	n.d
Rhizosediment of *J. maritimus*	53 ± 1	5.3 ± 0.1	58 ± 5	36 ± 5	6 ± 1	0.2 ± 0.2	n.d
Rhizosediment of *P. australis*	62 ± 2	5.7 ± 0.4	64 ± 3	31 ± 4	5 ± 3	0.1 ± 0.1	n.d

n.d: not detected

6.3.2 Plant endurance and root biomass

After one month, *J. maritimus* and *P. australis* plants presented stress symptoms. These results seem to be an effect of transplantation, since no differences among vases with and without petroleum were noted. By the end of the experiment, *J. maritimus* plants stems in the vases without the bioremediation treatments looked greener than those with BS and BA. On the other hand, no apparent differences among *P. australis* stems in uncontaminated and petroleum contaminated sediments, and with or without bioremediation treatments were found.

As observed for stems, at the end of the experiment, *J. maritimus* belowground tissues from uncontaminated sediment and from petroleum contaminated sediments without bioremediation treatments presented greater roots systems and slightly higher biomass (*e.g.* Figure 6.2-A) than plants subjected to BS and BA treatments (*e.g.* Figure 6.2-B). Visually, no differences were observed in *P. australis* belowground tissues, with roots and rhizome biomass remained similar in all vases, with no significant increase ($P > 0.05$) comparing with the initial belowground biomass (Figure 6.2-C). Although *P. australis* belowground biomass in BS treatment tended to be higher, this value was influenced by an outlier in one of the replicates.

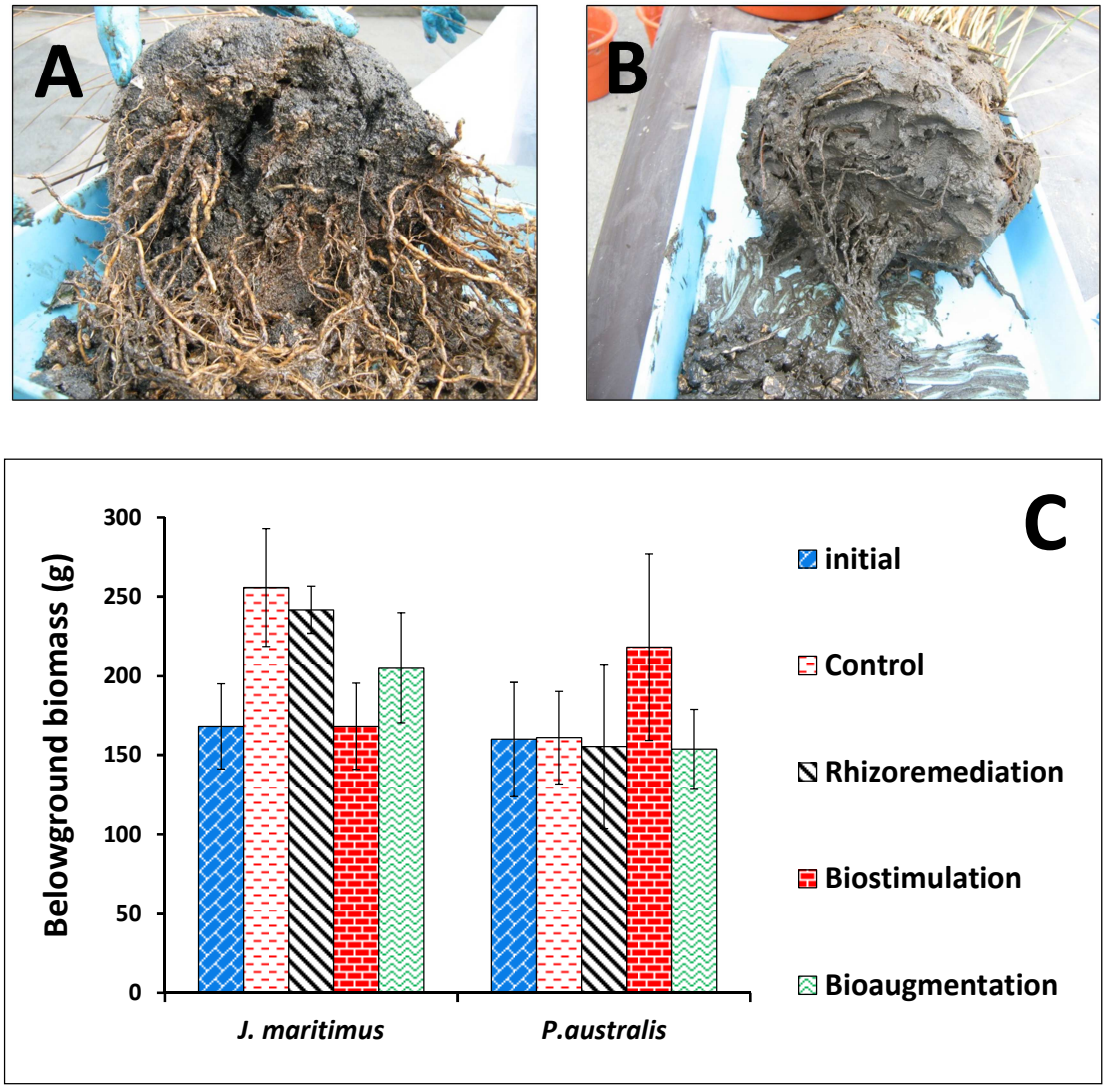

Figure 6.2 - **A)** Example of the root system of *Juncus maritimus* from rhizosediment without bioremediation treatments. **B)** Example of the root system of *J. maritimus* from rhizosediment with bioremediation treatments. **C)** Belowground biomass of *J. maritimus* and *Phragmites australis* before (initial) and after the 150 days experiment in un-contaminated sediments (Control) and crude oil contaminated sediments under different treatments.

6.3.3 Hydrocarbon degrading microorganisms abundance

Initial (T_0) HD abundance was significantly ($P < 0.05$) higher in rhizosediments than in un-colonized sediments (Figure 6.3), however without significant ($P > 0.05$) differences between *J. maritimus* and *P. australis* rhizosediments. The same trend was observed after five months (T_{150}), in the uncontaminated sediments and in contaminated sediments without bioremediation treatments.

However, in contaminated sediments with BS or with BA, treatment no significant ($P > 0.05$) differences were found among HD counts in colonized and un-colonized sediments. At T_{150}, HD abundance remained identical ($P > 0.05$) in uncontaminated sediment but increased in crude oil contaminated sediments as expected. The HD abundance in un-colonized sediments with BA treatment increased significantly ($P < 0.05$) comparing to uncontaminated sediments, although not significantly ($P > 0.05$) comparing with NA and BS treatment. However, in both rhizosediments, the highest HD abundance was observed in sediments without bioremediation treatments (RR treatment). Curiously, BS treatment did not increase significantly ($P > 0.05$) the HD abundance relatively to uncontaminated sediments and, in general, presented lower HD counts than RR and BA treatments.

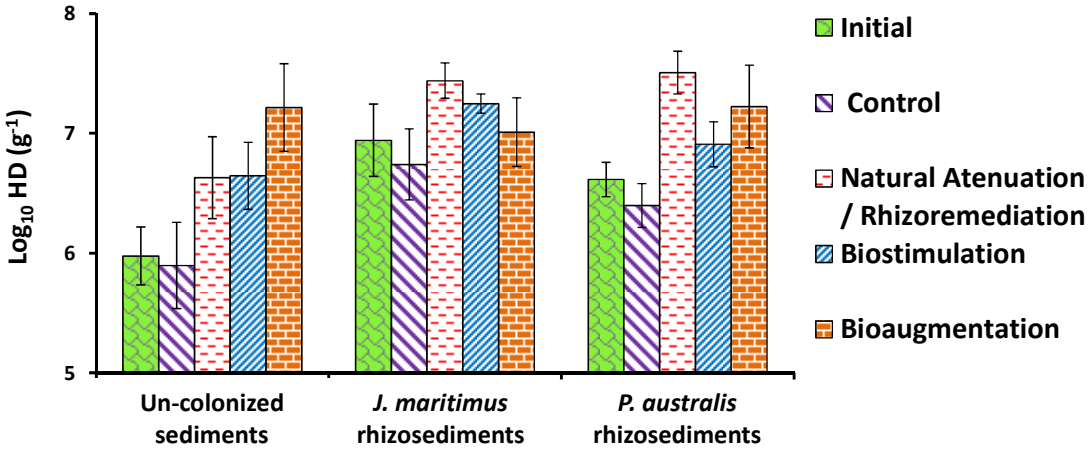

Figure 6.3 - Hydrocarbon degrading microorganisms estimated by Most Probable Number ($\log_{10}$ HD g^{-1}, mean and standard deviation, n = 3); in un-colonized sediments and in *Juncus maritimus* and *Phragmites australis* rhizosediments samples before (initial) and after the 150 days experiment in uncontaminated sediments (Control) and crude oil contaminated sediments under different treatments.

6.3.4 Potential of rhizoremediation and bioremediation treatments for hydrocarbon remediation in salt marsh sediments

To assess hydrocarbons degradation TPH concentrations were determined in all sediment samples (Table 6.2).

Table 6.2 - Total petroleum hydrocarbons (TPH) concentrations (conc.) (mg g^{-1}, mean and standard deviation, n = 3) in un-colonized sediments and rhizosediments of *Juncus maritimus* and *Phragmites australis* at the beginning (T_0) and after 150 days (T_{150}) in contaminated sediments. TPH concentrations in uncontaminated sediments (Initial) and the respective TPH degradation percentages are also shown (* $P < 0.05$ significant differences between T_0 and T_{150} samples).

	Initial sediments	Natural Attenuation / Rhizoremediation			Biostimulation			Bioaugmentation		
	T_0 TPH conc.	T_0 TPH conc.	T_{150} TPH conc.	Deg.	T_0 TPH conc.	T_{150} TPH conc.	Deg.	T_0 TPH conc.	T_{150} TPH conc.	Deg.
Un-colonized sediments	0.34 ± 0.06	6.1 ± 0.3	6.2 ± 0.2	**0 %**	6.4 ± 0.5	6.5 ± 0.4	**0 %**	6.7 ± 0.4	6.5 ± 0.6	**2.8 %**
J. maritimus rhizosediments	0.20 ± 0.04	7.3 ± 0.3	6.4 ± 0.2	***12 %**	6.8 ± 0.3	6.6 ± 0.2	**2.1 %**	7.1 ± 0.2	6.8 ± 0.3	**4.4 %**
P. australis rhizosediments	0.7 ± 0.1	8.1 ± 0.2	6.8 ± 0.5	***16 %**	7.9 ± 0.3	7.1 ± 0.3	***11 %**	7.9 ± 0.1	7.1 ± 0.1	***11 %**

In uncontaminated sediments, TPH concentrations after five months of experiment did not show a significant ($P > 0.05$) differences (results not shown), were similar to T_0 TPH concentrations. In un-colonized sediments also, no significant differences ($P > 0.05$) were observed between T_0 and T_{150} TPH concentrations in all treatments, although BA treatment seemed to induce a slight hydrocarbon degradation. For *J. maritimus*, RR treatment (12%) accomplished the higher hydrocarbons degradation in *J. maritimus* rhizosediments, being inclusively the only treatment that induced significant ($P < 0.05$) differences between T_0 and T_{150} TPH concentrations. *P. australis* rhizosediments presented the higher percentage of hydrocarbon degradation, accomplishing RR treatment the higher hydrocarbon degradation (16%), although BS treatment (11%) and BA treatment (11%) also presented significant ($P < 0.05$) differences between T_0 and T_{150} TPH concentrations.

GC-FID was used to obtain information on the profile of hydrocarbons in sediments, providing useful qualitative information of non-polar hydrocarbons within the range C_9H_{20}–$C_{40}H_{82}$ (Figure 6.4). In un-colonized sediments, for all treatments, differences between T_0 and T_{150} chromatograms were not visible. In *J. maritimus* rhizosediments, T_0 and T_{150} chromatogram of RR treatment also did not apparently showed differences, contradicting TPH data. It should be noted that TPH analysis can include more compounds than the present GC analysis.

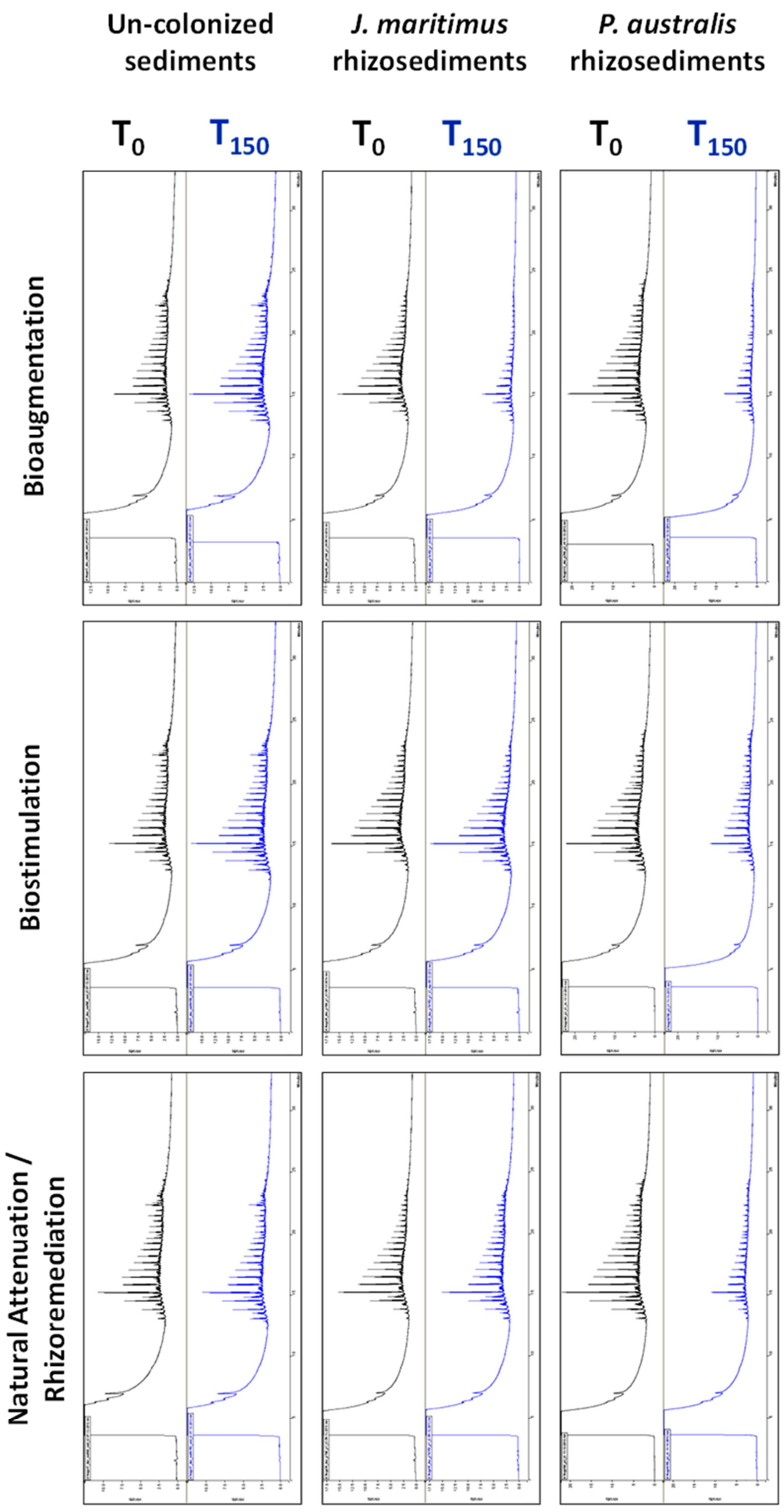

Figure 6.4 - Chromatograms of non-polar hydrocarbons within the range C_9H_{20} (n-nonane, retention time 9 min) -$C_{40}H_{82}$ (n-tetracontane, retention time 29 min) observed in crude oil contaminated un-colonized sediments and rhizosediments of *J. maritimus* and *P. australis* at the beginning (T_0) and at the end of the 150 days (T_{150}).

Additionally, no visible differences were found between T_0 and T_{150} chromatograms in *J. maritimus* rhizosediments with BS treatment. However, BA treatment contributed for *n*-alkanes degradation. In *P. australis* rhizosediments biodegradation effects were also visible in all treatments, with differences between T_0 and T_{150} chromatograms.

6.4 Discussion

The efficiency of rhizoremediation will depend on the establishment of a dynamic and synergistic relationship between plants and microorganisms (Wenzel, 2009), but also on the environmental conditions and plant characteristics. For rhizoremediation strategies, indigenous plants are preferred as they are well adapted to the environmental conditions and because it should also be intend to preserve native biodiversity. The introduction of foreign or genetically modified plant species can engender a serious ecological impact. The plants used in the present study, *J. maritimus* and *P. australis*, are common examples of ubiquitous species in European Atlantic and particular in Portuguese salt marshes (Adam, 2002; Costa et al., 2009a).

It is well recognized that oil spills may affect plants by decreasing plant height, stem density, and biomass, or causing complete mortality (*e.g.* Lin and Mendelssohn, 2009). Therefore, it is necessary to investigate if the chosen plants are suitable for hydrocarbon remediation. In our study, the final visual inspection suggest that both plant species were not affected by crude oil amendment, since no apparent visual differences were noted in aboveground tissues of plants transplanted in uncontaminated and petroleum contaminated sediments. Transplantation of adult plants was done to simulate a mature salt marsh. *J. maritimus* in BS and BA treatment also seems to be affected by the weekly nutrients solution addition, presenting lower root biomass than in the RR treatment. Root growth is highly influenced by environmental conditions, being highly responsive to nutrient availability and distribution within sediments (Hodge et al., 2009). According to Reynolds and D'Antonio (1996), root growth is generally favored in nutrient-poor soils. Indeed in our study, the lower availability of nutrients in vases without the bioremediation treatments

induced a functional response of *J. maritimus*, activating the root growth to explore a higher extent of sediments to enhance their nutrient acquisition. Therefore, BS and BA treatments, with their nutrient-rich environment appeared to delay the root development. In fact, López-Bucio et al. (2003) presented a similar effect in *Arabidopsis* roots, in which the increase nitrate availability reduced primary and lateral root elongation, being the number of lateral roots up to five times greater in plants grown in a limiting instead of an optimal nutrient concentration. Nevertheless, the response of root system to nutrients addition can be plant-specific, since no particular influence was found in *P. australis* root biomass.

As expected, salt marsh plants exerted a positive effect on the HD population in sediments initially collected, consistent with previous reports (Ribeiro et al., 2011; 2013a). In uncontaminated sediments after five months of the experiment, HD abundance was identical. Therefore, it was assumed that experimental conditions had no interference on the HD population. Moreover, the strong increase in HD abundance observed in crude oil contaminated sediments was a usual response of microbial adaptation and acclimation to the hydrocarbon contamination (Chaîneau et al., 2005). And, once more, HD counts were generally higher in rhizosediments, reflecting the "rhizosphere effect" (Olson et al., 2003).

The effectiveness of bioremediation has usually been evaluated by measurements of hydrocarbon degradation (Jensen et al., 2000). Low percentages of hydrocarbon degradation were achieved in vegetated sediments, suggesting that a longer time period may be required. Nevertheless, the presence of salt marsh plants clearly enhanced hydrocarbon degradation compared to the un-colonized sediments, for which degradation was negligible regardless of the applied treatments. In fact, significant hydrocarbon degradation and higher HD abundance was found in *J. maritimus* and *P. australis* rhizosediments, particularly with RR treatment, pointing to the potential of plant roots and their associated microorganisms for hydrocarbon removal. Curiously, *J. maritimus* root system seemed to have a direct effect on hydrocarbon degradation, as the higher HD counts and higher hydrocarbons degradation percentage was observed in the rhizosediments with the higher root biomass, which occurred in RR treatment. Nutrients increment had no additional or positive influence on hydrocarbon biodegradation in this study,

although *P. australis* roots system was not negatively influenced by nutrients addition. Under non-oxygen limiting conditions, laboratory studies suggest that adding nutrients may be effective for hydrocarbon removal in salt marsh sediments (*e.g.* Jackson and Pardue, 1999; Ribeiro et al., 2013b). However, crude oil biodegradation on marine wetlands is often limited by oxygen availability (Zhu et al., 2004). For instance, aeration is seldom a problem in sandy beaches, where BS or addition of marine organic substrate has been reported as successful strategy (*e.g.* Bragg et al., 1994; Mortazavi et al., 2013). In salt marshes, plants can transfer internal photosynthetic oxygen produced from the shoot system to roots, and released into rhizosphere (Holmer et al., 2002; Pezeshki and DeLaune, 2012). Plants with developed root systems, such as fibrous root systems, have been proposed for rhizoremediation of hydrocarbons in soils (Collins, 2007), because they can penetrate a larger soil volume, and have therefore a greater area for oxygen diffusion (Aprill and Sims, 1990; Lin and Mendelssohn, 1998). In fact, *P. australis* have a fibrous root system and presented higher hydrocarbon degradation than *J. maritimus* which has adventitious roots system born in a horizontal rhizome. We supposed that *J. maritimus* potential for hydrocarbons rhizoremediation depend on root system development, which was higher in low nutrient media. This may have led to an increase in oxygen diffusion and therefore to a higher hydrocarbon degradation. On the other hand, nutrients showed no influence on *P. australis* roots, concomitantly oxygen diffusion was analogous in all treatments, being hydrocarbon degradation similar among treatments.

Scrutiny of GC profiles can be useful to gain more insight about the hydrocarbons biodegradation susceptibility in salt marsh sediments. Confirming the previous results from TPH analysis, chromatograms did not show visible *n*-alkanes depletion in un-colonized sediments, reinforcing the idea that BS and BA treatments were insufficient to remediate hydrocarbon contaminated un-colonized sediments. On the other hand, RR treatment with *P. australis*, proved to be an effective strategy for hydrocarbon remediation, since *n*-alkanes biodegradation was observed, indicating that plant-microorganisms interaction has potential for remediating hydrocarbon contaminated sediments in salt marshes.

In the case of *J. maritimus*, chromatograms of rhizosediments clearly showed that the hydrocarbons profile was different among treatments. Surprisingly, in

the RR treatment *n*-alkanes depletion was not observed although TPH concentrations measurements showed a decrease after 5-months. Although it is generally accepted that *n*-alkanes are the most biodegradable hydrocarbons (Atlas, 1981), we supposed that another fraction, other than *n*-alkanes, but included in the TPH analysis were degraded. Several studies (*e.g.* Greenwood et al., 2008; Jones et al., 1983) evidenced that more resistant hydrocarbons can be degraded prior to *n*-alkanes, a situation that, according to these studies, can occur under aerobic and low nutrient conditions, that initially were supposed to exist in vases with *J. maritimus* rhizosediments without bioremediation treatment. Nevertheless, in our study, the BS treatment in *J. maritimus* rhizosediments proved to be, even to *n*-alkanes, an unsuccessful strategy, since chromatograms were similar at the beginning and at the end of the experiment. However, BA seems to potentiate *n*-alkanes degradation in *J. maritimus* rhizosediments. HD are ubiquitous in nature (Rosenberg, 2006), and enriched indigenous microorganisms of the contaminated site, that are potentially capable of degrading crude oil, is a much more promising technique than BS (Hosokawa et al., 2009). The fact that we used an enriched microbial consortium of each sediment type, rather than a pure culture, provided a metabolic diversity and robustness needed for field applications (Tyagi et al., 2011). However, the effectiveness of microbial amendments has not been convincingly demonstrated in the field (Zhu et al., 2004), although results from this study suggested that it can be a promising strategy to initiate and potentiate *n*-alkanes degradation pathways.

The information obtained in this study may be useful for the decision on which bioremediation strategies may be adequate to a specific contaminated salt marsh. One of the most important mechanisms for remediation of hydrocarbons contaminated sediments in wetlands seems to be based on the synergy of plant roots and microorganisms. Overall results suggest that plants can have an important role in restoration and remediation of oil-impacted salt marsh sediments. Reducing the effect of oxygen limitation seems to be the main role of plants in the hydrocarbon remediation, as they oxidize the rhizosphere (*e.g.* Howes et al., 1981), creating conditions to fostering aerobic HD and enhance hydrocarbon degradation. If oxygen is limiting, it can be assumed that higher root biomass will mean a larger rhizosphere for microbial stimulation in oxidized sediments. So, salt marsh rhizoremediation studies

should point to nutrients requirement that potentiate plant root development. However, nutrients availability can interfere with root biomass development as observed in the present work for *J. maritimus*. Therefore, the addition of nutrients can be counterproductive. Also, salt marshes located in or near estuaries are likely to be rich in nutrients; therefore, careful monitoring of background concentrations of nutrients can influence decisions to proceed with BS treatment.

A lack of knowledge regarding the role of the root system in hydrocarbons rhizoremediation is still evident, and understanding the dynamics of salt marsh plants roots may improve our knowledge and prediction of hydrocarbon degradation occurring in these ecosystems.

6.5 Conclusion

This work highlights to the role of plants in the hydrocarbon remediation in salt marshes, and to the specificity of each plant in terms of nutritional requirements for phytoremediation. Nutrients may not be the main limitation factor for hydrocarbon remediation in wetlands environment. Moreover, the addition of nutrients could, for some plants, decrease the hydrocarbon degrading ability of the plant-microorganism association, due to a counter-effect on the root system development. These findings can assist our understanding on the mechanisms of plants to influence hydrocarbons remediation.

Chapter 7

General discussion, considerations and future directions

General discussion, considerations and future directions

7.1 General discussion

The work described in this thesis focus on the evaluation of salt marsh plants potential for the rhizoremediation of PHC contaminated estuarine sediments. The impetous for this research, arised from the fact that little information is available on rhizoremediation on European estuaries. The choosen estuary – Lima located in NW of the Iberian Peninsula could be used as a template for other Atlantic temperate European estuaries. A successful rhizoremediation strategy requires first and foremost plant species that can survive in the habitat (*i.e.* sediments characteristics and climatic conditions) of the contaminated site. In the initial stage of the study, plants were choosen according to the diversity and abundance of the target salt marsh (Chapter 1). Based on these criteria, *J. maritimus*, *P. australis*, *T. striata* and *S. patens* (Chapter 2) were considered for this study.

The obtained results showed that salt marsh plants can, indeed positively influence the microbial community by increasing total microbial abundance, and promote the development of HD populations on its rhizosphere (Chapter 2 and 4). This assumption corroborates the "rhizosphere effect" (Olson et al. 2003) described for terrestrial environments. Greater bacterial counts in the rhizosphere including specific organisms capable of metabolizing a contaminant of concern are considered significant to plant-fostered biodegradation (Liste and Prutz, 2006). In fact, differences in degradation potentials were confirmed by means of laboratory experiments, which showed higher TPH degradation rates in rhizosediments than in un-colonized sediments (Chapter 2 and 5). Moreover, plants may be responsible for the movement of compounds into the rhizosphere, and contribute to a higher PHC retention at the vicinity of plant roots (Chapters 2 and 4).

With the underlying assumption that sediments natural characteristics affected PHC concentration (Chapter 2), research testing the characteristics influence on hydrocarbon degradation was assessed in rhizosediments colonized by the

same plant (Chapter 3). Distinct natural rhizosediments characteristics were assessed in four sites colonized by *J. maritimus*. Chapter 3 showed that rhizosediments particle size (Silt + Clay) and OM content were the variables that mainly contributed to hydrocarbons distribution within the salt marsh. In fact, in terrestrial environments, these characteristics have been considered to play a critical role in sorption/desorption and biodegradation processes (*e.g.* Xia and Wang, 2008). Moreover, the rhizosphere bacterial composition is likely to be affected by sediment characteristics (*e.g.* Marschner et al., 2004), and indeed, in Chapter 3 differences among the total microbial and specific HD abundance as well as among the microbial community structures of the different rhizosediments were found. ARISA profiles revealed a clear division among the microbial community structure that inhabited the four sites, even though sediments were colonized by the same plant. This sediment-specific change in microbial populations seems to affected degradation of PHC in contaminated rhizosediments. On one hand, coarser particles seem to provide more favorable conditions for the growth of HD eventually due to high rates of oxygen diffusion; on the other hand, hydrocarbons can be more accessible and available in coarser aggregates than on Silt + Clay particles (*e.g.* Brady and Weil, 1996; Otten et al., 1997). In fact Silt + Clay and OM content seemed to be the most influent factors in TPH degradation rates. The evidences described above suggested that rhizosediments characteristics could influence rhizosphere bacterial composition as well as the distribution and bioavailability of hydrocarbons with consequences for the rhizosphere hydrocarbon degradation microbial potential.

The subsequent step was to investigate the influence of distinct salt marsh plant species on PHC degradation potential. As stated earlier, *J. maritimus*, *P. australis* and *T. striata*, found in the same site colonizing sediments with similar characteristics (Chapter 2), were considered suitable candidates for further studies (Chapter 4 and 5). When designing cost-saving rhizoremediation strategies, plant capabilities to foster microbial numbers in their rhizosphere may be a useful tool (Chapter 2). During a phenological cycle, research testing the influence of the three plants on HD abundance was assessed (Chapter 4). Plants seemed to have a distinct influence on HD abundance particularly in what roots are concerned. Indeed, higher HD abundance was found in rhizosediments of the fibrous roots plants (*P.*

australis and *T. striata*), rather than in rhizosediments of the adventitious roots plants (*J. maritimus*). Fibrous roots systems can be an advantage since foster a larger rhizoplane surface area for microbial development and interactions, as well as a greater area for oxygen diffusion, stimulating aerobic HD. In fact, plants with fibrous root systems have been proposed for phytoremediation of hydrocarbons in soils (Collins, 2007), and have been related to higher degradation of petroleum hydrocarbons (Merkl et al., 2005).

Moreover, higher HD was found during spring, the flowering season for the target plants (Chapter 4). Interestingly, during the phenological cycle, a declining trend for TPH concentrations to decline during the periods of higher plant activity was encoraged by an increase of HD, showing an increased rhizoremediation activity. Although hydrocarbons inputs and outputs were not controlled, because Lima sampling site was an open system, this variation was probably due to plant-microorganisms interactions. Several factors sustain this assumption: higher rhizosediment HD (Chapter 4); diverse population of hydrocarbon degrading bacteria (Daane et al., 2001); oxidized rhizosediments due to roots respiration (Caffrey and Kemp, 1991), which supports microbial aerobic degradation processes; and finally, the plant influence on the movement and retention of TPH around their belowground tissues (Chapter 2 and 4). This is valuable information in order to predict the most adequate phase for rhizoremediation, which is less well characterized in the literature for soils and sediments.

Another factor theoretically contributing to the rhizoremediation potential was the plant-specific changes in microbial populations in the root environment (Segura and Ramos, 2012). It is well known that microbial community composition could be determined by several factors, including sediment characteristics (Chapter 3). Root exudates play a key role in plant–microorganism interactions by influencing the structure of soil microbial communities (Shi et al., 2011). It is becoming clear that salt marsh plants may influence the microbial composition in rhizosphere. Chapter 5 indicated that the presence/absence of plants and the plant species were the two major factors for determining bacterial community structure, being followed by others, such as petroleum amendment and nutritional conditions. Nevertheless, little is known in what way microbial communities respond to PHC contamination. Contamination by oil is generally expected to reduce the

biodiversity of the soil microbiota (Atlas et al., 1991), and eventually compromise their ecological functions. Therefore, understanding the microbial ecology and its constraints on rhizoremediation of an oil contaminated site is extremely important, since the reliable use of microbes carrying degradative pathways often suffers from a lack of information on their diversity, survival and metabolic activity under different environmental conditions (Galvão et al., 2005; Head et al., 2006; Lovley, 2003). Distinct salt marsh microbial communities responded in the same way to the petroleum contamination, *i.e.* increasing microbial abundance and changing microbial community structure (Chapter 5). Moreover, results showed that, with exception of *T. striata* rhizosediments, richness and diversity decreased in the petroleum-contaminated samples as expected, compared with sediments without petroleum amendment, particularly in samples with additional nitrogen. On one hand, richness decrease suggests a deleterious effect of added hydrocarbon in the most sensitive species, as previous reported by Grötzschel et al. (2002). On the other hand, we hypothesize that the main effect of petroleum amendment was the stimulation of HD, increasing the relative importance of these bacteria within the assemblage, which led to the obtained diversity reduction. Curiously, microbial community structure in *T. striata* and *P. australis* rhizosediments presented high similarities (above 65%) between treatments with and without petroleum, pointing to an apparent resistance to the disturbance. This resistance could be related with the specificity of the associated plant root system, since *T. striata* and *P. australis,* unlike *J. maritimus*, present a fibrous and dense root system as stated earlier. In fact, Chapter 4 highlighted that rhizosediments of these plants showed higher HD counts, and the highest TPH concentrations. Therefore, we hypothesize that the microbial community was familiarized with hydrocarbons, and not greatly impacted with further petroleum additions. Nevertheless, additional studies are needed to understand the significance of microbial community shifts associated with diversity decrease on their ecological functions.

It is well known that the efficiency of hydrocarbon rhizoremediation will depend on the establishment of a dynamic and synergistic relationship between plants and microorganisms (Wenzel, 2009), but also due to environmental conditions (Chapter 3), and plant characteristics (Chapter 4). In what rhizoremediation strategies were concerned, indigenous plants over

introduced including genetically modified should be preferred for two reasons (i) first they are well adapted to the environmental conditions; and (ii) second, they allow the preservation of native biodiversity. This is one of the reasons that the exotic *T. striata* (Chapter 4 and 5) was excluded from the mesocosms experiments (Chapter 6). The plants used in these experiments, *J. maritimus* (native and dominant) and *P. australis* are common examples of ubiquitous species in the Portuguese salt marshes (Chapter 1). Both plants were selected based on the potential for hydrocarbon degradation of microorganisms associated to theirs roots (Chapter 5), and the different root system (Chapter 4). Moreover, the period for the experiment duration (Chapter 6), *i.e.* April to September, was based in findings obtained in Chapter 4 that pointed to the plants ability to foster HD during the vegetative period of the phenological cycle.

Therefore, the aim of Chapter 6 was to assess the suitability of two salt marsh plants for PHC rhizoremediation, and additionally evaluate the efficiency of two bioremediation strategies, BS and BA. Experimental setup was conducted to mimic conditions in a real estuarine system by means of an automated irrigation system to simulate tidal dynamics, but also N and P concentrations identical to those in the water flooding the location where plants were collected. Results confirmed that salt marsh plants exerted a positive effect on the HD population, consistent with Chapter 2 and 4. Additionally, results highlighted rhizoremediation as an effective strategy for hydrocarbon removal, since hydrocarbon degradation in un-colonized sediments was negligible regardless the bioremediation treatments applied. Moreover, bioremediation treatments did not enhance the rhizoremediation potential of *P. australis*, and only BA treatment seemed to potentiate *n*-alkanes degradation in sediments colonized by *J. maritimus*. These results pointed to oxygen, and not nutrient availability, as the main factor affecting PHC degradation in salt marshes, an outcome also reported by other studies (*e.g.* Tate et al., 2012). However, under non-oxygen limiting conditions, laboratory studies suggested that adding nutrients may be effective for hydrocarbon removal in salt marsh sediments (Chapter 5). Therefore, since plants can oxidize rhizosphere (*e.g.* Holmer et al., 2002; Howes et al., 1981) they can have an important role in restoration and remediation of oil-impacted salt marsh sediments.

Curiously, the addition of nutrients seemed to influence *J. maritimus* root development, leading to a lower root biomass, which had a direct effect on hydrocarbon degradation potential (Chapter 6). López-Bucio et al. (2003) reported that the increasing nitrate availability reduced primary and lateral root elongation, being the number of lateral roots up to five times greater in plants grown in a limiting instead of an optimal phosphate concentration. It was supposed that *J. maritimus* potential for hydrocarbons rhizoremediation depended on root system development, which was higher in low nutrient media. Higher root system may have led to an increase in oxygen diffusion and therefore to a higher hydrocarbon degradation. On the other hand, nutrients showed no influence on *P. australis* roots, concomitantly oxygen diffusion was analogous in all treatments, being hydrocarbon degradation similar among treatments. Moreover, *P. australis* with a fibrous root system presented higher hydrocarbon degradation than *J. maritimus* which has adventitious roots, but are dominat in the Lima estuary salt marshes. This strengthens the idea that plants with fibrous root systems had higher hydrocarbon degradation potential (Chapter 4).

These findings are important to elucidate the mechanisms of plants to influence hydrocarbon remediation. Nevertheless, more insights on plant-microorganisms interactions are needed, in particular among salt marsh plant species, in order to fully ascertain the interactions of rhizosphere on hydrocarbon biodegradation.

7.2 Final considerations and future directions

Understanding the influence of different salt marsh plants on the fate and remediation of PHC can be useful to improve the performance and acceptance of friendly environment strategies, such as rhizoremediation rather than the traditional more invasive technologies that often cause more harm than good from the environmental point of view. The present work focused on plants found in a temperate Atlantic estuarine environment (NW Portugal), and therefore with wider geographical applicability. The major outcomes of this research were:

- Plants influence the microbial community, by fostering the development specific HD populations and increasing total microbial abundance in its rhizosphere. Distinct plants have different influence on the dynamics of HD populations, which seemed to be markedly thriving by plants with fibrous root morphology (*P. australis* and *T. striata*). Salt marsh plants with fibrous roots (*e.g. P. australis* and *T. striata*) tend to influence distribution and retention of hydrocarbons around their belowground tissues more efficiently than plants with adventitious root system (*e.g. J. maritimus*). These outcomes are especially highlighted in seasons of higher plant activity.

- Natural sediment characteristic can influence rhizosphere bacterial composition as well as the distribution and bioavailability of hydrocarbons with consequences for the rhizosphere hydrocarbon degradation microbial potential. Coarser particles with low OM content seem to provide more favorable conditions for the growth of HD, and for higher TPH degradation.

- When exposed to petroleum contamination, plant species emerged as the major factor for shaping the rhizosphere community structure, overriding the petroleum and nutrients influence. Moreover, distinct salt marsh microbial communities responded similarly with (i) increased abundance, (ii) changes in structure; and (iii) decreased diversity to petroleum contamination. With the quantitative changes, ARISA analysis showed a qualitative shift on salt marsh bacterial community structure and diversity. Ecologically, bacterial community associated to plants with fibrous roots appeared to be more resistant to petroleum contamination than those from plants with adventitious roots.

- Laboratory and greenhouse experiments showed that microbial communities associated to *J. maritimus* and *P. australis* roots displayed a potential to degrade petroleum hydrocarbons in salt marsh environment. It should be noted that hydrocarbon degradation in un-colonized sediments was negligible regardless the bioremediation treatments (BS and BA) applied. Bioremediation treatments did not enhance the rhizoremediation potential of *P. australis*, and only BA treatment seemed to potentiate *n*-alkanes degradation

in sediments colonized by *J. maritimus*. These results highlight that nutrients may not be the main limitation factor affecting PHC degradation in salt marshes. These studies also alert to the specificity of each plant in terms of nutritional requirements for phytoremediation, as nutrient amendments can eventually decrease the hydrocarbon degrading ability of the plant-microorganism association, due to a counter-effect on the root system development.

These findings can assist our understanding on the mechanisms of plants to influence hydrocarbons remediation, and should be considered when designing rhizoremediation strategies in estuaries. However, additional studies are in dear need; particularly regarding the mechanisms responsible for the observed plant-specific rhizoremediation outcomes. These future directions can be summarized as follows:

- ✓ Field surveys (*in situ*) to confirm findings from laboratory results (*ex situ*). What other biotic and abiotic factors controlling the growth and metabolic activities of plant-microorganisms interactions in PHC polluted salt marshes are important?

- ✓ A lack of knowledge regarding the role of the root system in hydrocarbons rhizoremediation is still evident. Are plant roots in fact critical to the observed effects? What is the effect of the root system on the pattern and quantity of oxygen released to the rhizosphere? If oxygen is a limiting factor in salt marsh ecosystems, how to enhance the oxygen supply?

- ✓ Which microbial strains appeared in oiled sediments? What is the significance of microbial community shifts associated with diversity decrease on ecological function and impact on PHC degradation? The study of resilience, *i.e.* the rate at which microbial composition returns to its original composition after being disturbed, could be a useful and sensitive way of monitoring the impact and recovery of petroleum-contaminated sediments?

- ✓ What are the plant-specific induced responses to hydrocarbon contamination, at the beginning and during the rhizoremediation process? If nutrient availability is a limiting step, where do N and P come from in the presence of plants to allow for improved rhizoremediation compared with un-colonized sediments? What exudates are being deposited into the rhizosphere during the remediation process that helps PHC degradation?

To answer all these questions, and to successfully implement phytoremediation technology, a multidisciplinary approach and basic knowledge in microbiology, biochemistry, physiology, ecology and genetics are required. Information that can be derived from these studies may provide further insights on how to design a successful rhizoremediation strategy.

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