Universidade de Aveiro Departamento de Biologia
Ano 2014

Vanessa Jesus Oliveira

Mediação microbiana na recuperação de sapais contaminados com hidrocarbonetos

Microbe-mediated recovery of salt marshes contaminated with oil hydrocarbons

Universidade de Aveiro Departamento de Biologia
Ano 2014

Vanessa Jesus Oliveira

Mediação microbiana na recuperação de sapais contaminados com hidrocarbonetos

Microbe-mediated recovery of salt marshes contaminated with oil hydrocarbons

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ângela Cunha, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro, do Professor Doutor Artur Silva, Professor Catedrático do Departamento de Química da Universidade de Aveiro e do Doutor Newton Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM).

Este trabalho foi finaciado pelo Centro de Estudos do Ambiente e do Mar *(CESAM) (*PEst-C/MAR/LA0017/2011), pela unidade de investigação QOPNA (project PEst-C/QUI/UI0062/2011) e pela Fundação para a Ciência e Tecnologia (FCT) project PTDC/AAC-CLI/107916/2008 e COMPETES-(FCOMP-01-0124-FEDER-008657).

Vanessa Jesus Oliveira recebeu apoio financeiro da Fundação para a Ciência e Tecnologia (FCT) e do Fundo Social Europeu (FSE) no âmbito do III Quadro Comunitário de Apoio sobre a forma de Bolsa Individual de Doutoramento (SFRH/BD/46977/2008).

o júri

presidente

Professor Doutor Manuel João Senos Matias
Professor Catedrático do Departamento de Geociências da Universidade de Aveiro

Professor Doutor António Carlos Matias Correia
Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

Professora Doutora Maria Isabel Violante Caçador
Professora Auxiliar com Agregação da Faculdade de Ciências da Universidade de Lisboa

Professora Doutora Maria Ângela Sousa Dias Alves Cunha
Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Doutor Rodrigo da Silva Costa
Investigador Auxiliar do Centro de Ciências do Mar da Universidade do Algarve

Doutora Catarina Maria Pinto Mora Pinto de Magalhães
Investigadora auxiliar do Centro Interdisciplinar de Investigação Marinha e Ambiental – CIIMAR, Porto

agradecimentos

Gostaria de agradecer a todas as pessoas que me ajudaram a tornar possível a realização deste trabalho:
À Professora Doutora Ângela Cunha, orientadora desta tese, o meu profundo agradecimento pelo excecional apoio, confiança, e toda a disponibilidade no decurso deste trabalho.
Ao Doutor Newton Gomes, co-orientador desta tese, o meu sincero agradecimento, pelo interesse, apoio e ideias que contribuiram grandemente para o resultado final deste trabalho.
Ao Professor Doutor Artur Silva, co-orientador desta tese, o meu sincero agradecimento pelo interesse, pela disponibilidade e sobretudo pela forma com que sempre me recebeu.
Ao Professor Doutor Mário Simões, pela permanente disponibilidade, paciência e incansável acompanhamento de uma Bióloga no mundo da Química.
Á Doutora Kornelia Smalla, e todo o seu grupo do Julius Kühn-Institut, Braunschweig, Alemanha, pela forma que me receberam no mini-estágio que realizei no início deste trabalho e o seu contributo enriquecedor para um dos capítulos deste trabalho.
À Professora Doutora Adelaide Almeida, Professora Doutora Helena Silva e ao Professor Doutor João Serôdio pelo incentivo e colaboração.
À Doutora Ana Lillebø e Doutor Daniel Cleary pela abertura à colaboração, pelo apoio científico e pelos valiosos ensinamentos transmitidos.
Ao João Ezequiel e Samuel Oliveira pela preciosa contribuição no trabalho laboratorial.
À minha amiga e colega Ana Luísa Santos, pelas viagens, aventuras e por me apoiar sempre mesmo estando longe.
À minha amiga e colega Inês Baptista, pelas companhia e ajuda na recolha das amostras em campo, por vezes de madrugada e por toda a sua boa disposição.
Aos elementos dos laboratórios de Microbiologia Ambiental e Aplicada e Ecologia Molecular e Ambientes Marinhos, por contribuírem para um ambiente de trabalho agradável e todo o apoio tanto nos bons como menos bons momentos……
À Universidade de Aveiro, por fornecer as instalações para a realização deste trabalho.
À Fundação para a Ciência e Tecnologia (FCT) e CESAM (Centro de Estudos do Ambiente e do Mar) pelo financiamento.
A todos os meus amigos e à minha grande familia, principalmente aos meus primos por estarem sempre presentes e me ajudarem a descontrair.
Finalmente, aos meus pais e irmã, as pessoas mais importantes da minha vida neste momento, por todo o apoio, paciência e principalmente pela confiança que sempre tiveram em mim.

palavras-chave

Sapais, hidrocarbonetos, interacção planta/bácteria, bactérias endofíticas,fitoremediação

resumo

As zonas de sapal são ambientes intertidais altamente produtivos, que servem como áreas de reprodução para muitas espécies com grande importância a nível comercial e económico. Devido à sua localização e às suas características físicas e biológicas, os sapais são particularmente susceptíveis à exposição a hidrocarbonetos com origem antropogénica. A contaminação dos sedimentos com hidrocarbonetos de petróleo é especialmente nociva para a vegetação de sapal, uma vez que os hidrocarbonetos aromáticos de baixo peso molecular podem afectar todos os estágios de desenvolvimento das plantas. No entanto, a utilização de plantas para biorremediação (fitorremediação) por remoção ou captura de compostos tóxicos, tem sido amplamente estudada. A fitorremediação é encarada como uma abordagem eficiente, económica e de baixo impacto ambiental para remoção de hidrocarbonetos aromáticos, que envolve a intervenção directa das plantas coadjuvada pela atividade das populações microbianas degradadoras na rizosfera (fitorremediação assistida por microrganismos). Nas rizosferas desenvolvem-se comunidades de microorganismos equipados com genes catabólicos relacionados com a degradação de hidrocarbonetos do petróleo (OH), com potencial na destoxificação do sedimento em torno destas raízes. Além disso, uma vez que algumas bactérias da rizosfera são também capazes de colonizar os tecidos das plantas (bactérias endofíticas), designadamente da raiz, as comunidades rizocompetentes degradadoras de OH são importantes para a degradação de OH ″in planta“ e contribuem para o seu papel como agentes de biorremediação.

Este trabalho envolveu uma componente de campo e uma compoente laboratorial desenvolvida em microcosmos, com o objectivo de caracterizar interacções planta-bactéria relevantes na bioremediação de sapais contaminados com hidrocarbonetos e optimizar combinações planta-bactéria para uma melhoria da sobrevivência das plantas e aceleração da degradação dos poluentes.

Na abordagem de campo, foram utilizadas ferramentas moleculares para avaliar como o tipo de espécie de planta e a contaminação com hidrocarbonetos afetam a composição das comunidades bacterianas do sedimento [sedimentos sem vegetação e sedimentos em torno das raízes (rizosfera) das espécies *Halimione portulacoides* e *Sarcocornia perennis subsp. perennis*] num estuário temperado (Ria de Aveiro, Portugal) cronicamente exposto à poluição por OH.

As sequências de genes de rRNA 16S obtidas neste estudo foram usadas para gerar metagenomas "*in silico*" e inferir tendências nos perfis funcionais das comunidades bacterianas em diferentes microhabitats. Posteriormente, uma combinação de métodos dependentes e independentes de cultivo foi utilizada para investigar o efeito de contaminação com OH sobre a estrutura e função da comunidade bacteriana endófitica das halófitas. Os sistemas radiculares de *H. portulacoides* e *S. perennis* subsp. *perennis* parecem exercer uma forte influência sobre a composição bacteriana e a análise metageomica "*in silico*" revelou um enriquecimento em genes envolvidos no processo de degradação de hidrocarbonetos aromáticos policíclicos (PAHs) na rizosfera de plantas halófitas. Na fracção cultivável de endofíticas degradadoras foram detetadas com particular frequência, espécies de *Pseudomonas* conhecidas como degradadoras de OH. As comunidades endofíticas revelaram um efeito local, relacionado com características do sedimento, tal como, o nível de contaminação de OH.

A fim de determinar se a inoculação da *H. portulacoides* com bactérias degradadoras de hidrocarbonetos podem mitigar os efeitos negativos da exposição das plantas à contaminação por hidrocarbonetos, bem como avaliar as respostas em termos de estrutura e função das comunidades bacterianas associada à raíz de plantas (rizosfera e endosfera), foi desenvolvida uma experiência de microcosmos. A halófita *Halimione portulacoides* foi inoculada com uma estirpe de *Pseudomonas* sp., uma bactéria endofítica degradadora previamente isolada, e cultivada em sedimentos experimentalmente adicionados com 2-metilnaftaleno. Embora os resultados não tenham demonstrado um efeito significativo do contaminante sobre a condição da planta, a redução da concentração de 2-metilnaftaleno no sedimento no final da experiência sugere que *H. portulacoides* pode ser considerada como uma planta com potencial interesse para aplicação na fitorremediação de zonas de sapal contaminadas com hidrocarbonetos aromáticos. Apesar da degradação do hidrocarboneto não ter sido acelerada pela inoculação das plantas com uma estirpe de *Pseudomonas* sp. degradadora, esta parece ter exercido um efeito positivo sobre a condição das plantas, independentemente da adição de 2-metilnaftaleno. Os efeitos da inoculação sobre a estrutura da comunidade endófitica observada no final da experiência indicam que a estirpe pode ser uma colonizadora eficiente das raízes da *H. portulacoides*.
Os resultados obtidos neste trabalho sugerem que a *H. portulacoides* tolera concentrações moderadas de 2-metilnaftaleno podendo assim ser considerada como um agente promissor para processos de fitoremediação em sapais contaminados com hidrocarbonetos de petróleo. As plantas suportam comunidades bacterianas endofíticas diversas e enriquecidas em fatores géneticos (genes) relacionados com degradação de hidrocarbonetos e as interações planta/bactéria podem assumir um importante papel nos processos de degradação.

keywords

Salt marshes, hydrocarbons, plant/bacteria interaction, endophytic bacteria, phytoremediation

abstract

Salt marshes are highly productive intertidal habitats that serve as nursery grounds for many commercially and economically important species. Because of their location and physical and biological characteristics, salt marshes are considered to be particularly vulnerable to anthropogenic inputs of oil hydrocarbons. Sediment contamination with oil is especially dangerous for salt marsh vegetation, since low molecular weight aromatic hydrocarbons can affect plants at all stages of development. However, the use of vegetation for bioremediation (phytoremediation), by removal or sequestration of contaminants, has been intensively studied. Phytoremediation is an efficient, inexpensive and environmental friendly approach for the removal of aromatic hydrocarbons, through direct incorporation by the plant and by the intervention of degrading microbial populations in the rhizosphere (microbe-assisted phytoremediation). Rhizosphere microbial communities are enriched in important catabolic genotypes for degradation of oil hydrocarbons (OH) which may have a potential for detoxification of the sediment surrounding the roots. In addition, since rhizosphere bacterial populations may also internalize into plant tissues (endophytes), rhizocompetent AH degrading populations may be important for *in planta* AH degradation and detoxification.

The present study involved field work and microcosms experiments aiming the characterization of relevant plant-microbe interactions in oil-impacted salt marshes and the understanding of the effect of rhizosphere and endosphere bacteria in the role of salt marsh plants as potential phytoremediation agents.

In the field approach, molecular tools were used to assess how plant species- and OH pollution affect sediment bacterial composition [bulk sediment and sediment surrounding the roots (rhizosphere) of *Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*] in a temperate estuary (Ria de Aveiro, Portugal) chronically exposed to OH pollution. In addition, the 16S rRNA gene sequences retrieved in this study were used to generate *in silico* metagenomes and to evaluate the distribution of potential bacterial traits in different microhabitats. Moreover, a combination of culture-dependent and -independent approaches was used to investigate the effect of oil hydrocarbons contamination on the structure and function of endophytic bacterial communities of salt marsh plants.

Root systems of *H. portulacoides* and *S. perennis* subsp. *perennis* appear to be able to exert a strong influence on bacterial composition and *in silico* metagenome analysis showed enrichment of genes involved in the process of polycyclic aromatic hydrocarbon (PAH) degradation in the rhizosphere of halophyte plants. The culturable fraction of endophytic degraders was essentially closely related to known OH-degrading *Pseudomonas* species and endophytic communities revealed site-specific effects related to the level of OH contamination in the sediment.

In order to determine the effects of oil contamination on plant condition and on the responses in terms of structure and function of the bacterial community associated with plant roots (rhizosphere, endosphere), a microcosms approach was set up. The salt marsh plant *Halimione portulacoides* was inoculated with a previous isolated *Pseudomonas* sp. endophytic degrader and the 2-methylnaphthalene was used as model PAH contaminant. The results showed that *H. portulacoides* health and growth were not affected by the contamination with the tested concentration. Moreover, the decrease of 2-methylnaphthalene at the end of experiment, can suggest that *H. portulacoides* can be considered as a potential plant for future uses in phytoremedition approaches of contaminated salt marsh. The acceleration of hydrocarbon degradation by inoculation of the plants with the hydrocarbon-degrading *Pseudomonas* sp. could not, however, be demonstrated, although the effects of inoculation on the structure of the endophytic community observed at the end of the experiment indicate that the strain may be an efficient colonizer of *H. portulacoides* roots.

The results obtained in this work suggest that *H. portulacoides* tolerates moderate concentrations of 2-methylnaphthalene and can be regarded as a promising agent for phytoremedition approaches in salt marshes contaminated with oil hydrocarbons. Plant/microbe interactions may have an important role in the degradation process, as plants support a diverse endophytic bacterial community, enriched in genetic factors (genes and plasmids) for hydrocarbon degradation.

"In the great battles of life, the first step to victory is the desire to win."

Mahatma Gandhi

Mediação microbiana na recuperação de sapais contaminados com hidrocarbonetos

Microbe-mediated recovery of salt marshes contaminated with oil hydrocarbons

List of Publications

This thesis includes results which have already been published in the journals listed below:

Oliveira V., Gomes N.C.M., Almeida A., Silva A.M.S., Silva H., Cunha A. (2014) Microbe-assisted phytoremediation of hydrocarbons in estuarine environments. *Microbial Ecology*, accepted. DOI: 10.1007/s00248-014-0455-9

Oliveira V., Gomes N.C.M., Cleary D.F.R. Cleary, Almeida A., Silva A.M.S., Simões M.M.Q., Silva H., Cunha A. (2014) Halophyte plant colonization as a driver of the composition of bacterial communities in salt marshes chronically exposed to oil hydrocarbons. *FEMS Microbiology Ecology*, accepted. DOI: 10.1111/1574-6941.12425

Oliveira V., Gomes N.C.M., Almeida A., Silva A.M.S., Simões M.M.Q., Smalla K., Cunha A. (2014) Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria. *Molecular Ecology* 23, 6, 1392-1404. DOI:10.1111/mec.12559

CONTENTS

List of Figures……1

List of Tables…… 4

Abbreviation List…….. 6

Thesis Outline……8

CHAPTER 1

Microbe-assisted phytoremediation of hydrocarbons in estuarine environments

Introduction……12

Hydrocarbons…….15

Phytoremediation of hydrocarbons……17

Microbial hydrocarbon degradation…….. 20

Microbe-assisted phytoremediation……...22

Current limitations to the microbe-assisted phytoremediation of hydrocarbons...26

Monitoring plant-bacteria interactions involved in microbe-assisted phytoremedition…….27

Future perspectives……28

Acknowledgments……29

CHAPTER 2

Halophyte plant colonization as a driver of the composition of bacterial communities in salt marshes chronically exposed to oil hydrocarbons

Introduction……... 31

Material and Methods

Sampling sites and sample processing……..33

Sediments properties…….34

Hydrocarbon analysis……34

Total community DNA extraction…… 35

PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE)……35

Barcoded-pyrosequencing…….36

Data analysis…….37

Phylogenetic tree…….. 38

In silico metagenome analysis…….. 38

Results and Discussion
Sediment properties and hydrocarbon contamination…….. 39
Site-related variation of the structure of microbial communities……. 43
Structure of bacterial communities in salt marsh sediments…… 44
Plant-related variation in the structure of bacterial communities…… 49
In silico metagenome analysis……..52
Conclusion…….53
Acknowledgements……54
Data Accessibility……..54
Supplementary Material…….55
CHAPTER 3
Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria
Introduction……58
Material and Methods
Experimental site and sampling……60
Sediment properties…….. 61
Endophytic communities
DNA extraction…… 62
DGGE analysis…….62
Detection of PAH degrading genes……. 63
Determination of gene copy number of PAH ring-hydroxylating dioxygenase genes of Gram-negative bacteria…….63
Endophytic isolates
Enrichment and isolation of PH-degrading bacteria…… 64
DNA extraction…… 65
Characterization and identification of endophytic strains……65
Detection of genes encoding for PAH degradation……. 66
PCR-detection of broad host range (BHR) plasmids…….. 66
Plasmid-DNA extraction and characterization…….66
Detection of genes encoding for PAH degradation enzymes in plasmidic DNA……66
Sequence analysis of PAH degrading genes and phylogenetic tree…….67
Statistical analysis……..67

Results and Discussion

Environmental variables and endophytic community composition……. 68

Plant species effects on endophytic bacterial communities……..69

PAH dioxygenase genes in total endophytic communities…….. 70

Endophytic bacteria enriched in selective cultures…….. 73

PAH genes and BHR plasmids in endophytic isolates……. 73

Conclusions……76

Acknowledgements……76

Data Accessibility……..77

Supplementary Material……78

CHAPTER 4

Evaluation of plant-microbe interactions in 2-methylnaphthalene contaminated sediments

Introduction……82

Material and Methods……83

Treatments……84

Quantification of 2-methylnaphthalene…… 84

Photosynthetic performance……. 85

Total community DNA (TC-DNA) extraction……. 86

PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE)……87

Detection of genes encoding for PAH degradation…….. 88

Statistical analysis……..88

Results

Growth, maximum quantum yield of PSII and light curve parameters…… 88

Concentration of 2-methylnaphthalene……89

DGGE analysis of the bacterial community……. 90

Detection of genes encoding for PAH degradation…….. 92

Discussion……..92

Plant condition…….. 92

Hydrocarbon clearance…… 93

Structure of bacterial communities…….. 94

Hydrocarbon degradation genes……95

Conclusions……96

Acknowledgements……96
Supplementary Material……98
CHAPTER 5
Conclusions……..100
Field work……...…100
Microcosm component……103
REFERENCES……105

List of Figures

Fig. 1.1 Sources and processes involved in hydrocarbon release into estuarine ecosystems……...17

Fig. 1.2 Plant-microbe interactions and plant-growth-promoting effects of rhizosphere and endosphere bacteria……..22

Fig. 2.1 Ria de Aveiro (Portugal) with the location of sampling stations (A, B, C or D)…….33

Fig. 2.2 Ordination based on principal coordinate analysis (PCO) of bacterial community profiles of different microhabitats (a, b, c) in each sampling sites (A, B, C and D)…….43

Fig. 2.3 Relative abundance of the most represented bacterial taxa: three most abundant phyla (a, b, c), six most abundant classes (e, f, g, h, i), the seven most abundant orders (j, k ,l, m, n, o, p, q) and the most dominant OTU (d) in bulk sediments (BS), *H. portulacoides* rhizosphere (RH) and *S. perennis* rhizosphere (RS)…….45

Fig. 2.4 Phylogenetic tree showing the *Proteobacteria* (A) and *Actinobacteria-Acidobacteria-Bacteriodetes* (B) clusters from bulk sediment and salt marsh rhizospheres of *H. portulacoides* and *S. perennis*. Close relatives of the selected sequences were obtained by using the BLAST search. H: Microhabitat (rhizosphere samples from *Halimione portulacoides* (Hal) and *Sarcocornia perennis subsp. perennis* (Sar)); S: Sampling site (A, B, C or D); RA: Relative abundance of the respective OTU…….47

Fig. 2.4 (continued)…… 48

Fig. 2.5 (a) Ordination based on principal coordinate analysis (PCO) of pyrosequencing data. Samples are represented by symbols (BS – Bulk sediment; RH – *H. portulacoides*

rhizosphere and RS – *S. perennis* rhizosphere). Operational taxonomic unit (OTU) are represented by circles and OTUs with > 150 sequences are represented by large circles. (b) OTU number of dominant 16S rRNA sequence reads (> 150 sequences)……50

Fig. 2.6 Relative gene count of selected KOs could possible involved in PAH degradation. Samples are represented by symbols (BS – Bulk sediment; RH – *H. portulacoides* rhizosphere and RS – *S. perennis* subsp. *perennis* rhizosphere). KOs enzyme terminology: K00449-pcaH; K0042 - 1.14.13.-:Oxidoreductases; K00517 - E1.14.-.-:Oxidoreductases; K00599 - E2.1.1.-: Transferases.…….53

Fig. 3.1 Ria de Aveiro (Portugal) with the location of sampling stations (A, B, C or D)…….60

Fig. 3.2 NMDS showing the comparison between DGGE fingerprints from endophytic community from two different plants, *Halimione portulacoides* (A) and *Sarcocornia perennis* subsp. *perennis* (B), along a gradient of hydrocarbons contamination……70

Fig. 3.3 Quantification of PAH-RDHα GN gene copy number contained in endophytic community in a gradient of hydrocarbons contamination (mean value ± STD, n = 4)……..71

Fig. 3.4 Phylogenetic relationships of amino acid sequences of genes encoding PAH degradation enzymes detected in bacterial isolates using protocol 1. The sequences were aligned with related sequences retrieved from GenBank……74

Fig. 3.5 Phylogenetic relationships of amino acid sequences of genes encoding PAH degradation enzymes detected in bacterial isolates using the set of primers of protocol 2. The sequences were aligned with related sequences retrieved from GenBank……...75

Fig. 4.1 Ordination based on principal coordinate analysis (PCO) showing the comparison between DGGE fingerprints from rhizosphere community in the four treatments (Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant)……91

Fig. 4.2 Ordination based on principal coordinate analysis (PCO) showing the comparison between DGGE fingerprints from endophytic community in the four treatments (Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant)……91

Supporting information

Fig. 4.S1 *Halimione portulacoides* inoculated with *Pseudomonas* sp. endophytic degrader……...98

Fig. 4.S2 Plant/sediment microcosm in the end of the experiment…… 98

List of Tables

Table 1.1 Concentration of various pollutants detected in sediments at estuaries from around the world……..14

Table 1.1 (continued)……..15

Table 1.2 Halophytes used for phytoremediation of hydrocarbons in estuarine areas...19

Table 1.3 Factors affecting the biodegradation of petroleum hydrocarbons……..21

Table 1.4 Examples of successful approaches of remediation of hydrocarbons by combined use of plants and rhizo- and/or endophytic bacteria…….. 25

Table 2.1. Sediment properties in four salt marshes sites of a estuarine system Ria de Aveiro (main value ± STD, n = 3)……. 39

Table 2.2. Concentration of aliphatic hydrocarbons (ng g^{-1} dry weight) and values of selected source diagnostic indices in four salt marsh sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3)……..40

Table 2.3 Concentration of PAH (ng g^{-1} dry weight) in four salt marsh sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3)……...41

Table 2.4 Origin-indicative PAH ratios and ratio values calculated for this study……42

Table 3.1 Primer and amplification conditions used for PCR amplification……..64

Table 3.2 Sediment properties and concentration of hydrocarbons in four salt marshes sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3)…….68

Table 3.3 Endophytic bacterial isolates from enrichment cultures of *H. portulacoides* and *S. perennis* subsp. *perennis* roots…….72

Table 4.1 Chlorophyll a fluorescence parameters initial and after eight weeks of 2-methylnaphthalene contamination. Maximum quantum yield of PSII (F_v/F_m) and light curve parameters (α, initial slope), $_rETR_m$ (maximum relative electron transport rate) and E_k, light-saturation) for *H. portulacoides*…….89

Table 4.2 Quantification of 2-methylnaphthalene (ng g dwt^{-1})……..90

Table 4.3 Detection of genes encoding PAH degradation enzymes in each treatment……..92

Supporting information

Table 2.S1. Estimators of sequence library diversity and coverage……...55

Table 3.S1 Detection of genes encoding PAH degradation enzymes on endophytic community from roots in a gradient of hydrocarbon contamination. -, no detection in all replicates; +, detection in one replicate; ++, detection in two replicates; +++, detection in three replicates (n = 4)…….78

Table 3.S2 PCR and SBH analysis of genes encoding PAH degradation enzymes involved in hydrocarbon degradation and plasmid groups of endophytic isolates. The signal + represents the number of isolates where genes were detected. - = hybridization and (-) = weak hybridization……...79

Abbreviation List

ACC 1-aminocyclopropane-1-carboxylate

AIC Akaike Information Criterion

BHR Broad host range

BSA Bovine serum albumin

COG Clusters of Orthologous Groups of proteins

DistLM Distance based linear models

DGGE Denaturing gradient gel electrophoresis

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DTT Dichlorodiphenyltrichloroethane

FISH Fluorescence *in situ* hybridization

GC-MS Gas chromatography mass spectrometry

HCB Hexachlorobenzene

HCH Hexachlorocyclohexane

HMW High molecular weight

JTT Jones-Taylor-Thornton

KEGG Kyoto encyclopedia of genes and genomes

KO KEGG orthologs

LMW Low molecular weight

MEGA5 Molecular Evolutionary Genetics Analysis 5

MPN Most-probable-number

NMDS non-metric multidimensional scaling

NNI Nearest neighbor interchange

OH Oil hydrocarbons

OM Organic matter

OTU Operational taxonomic units

PAH Polycyclic aromatic hydrocarbons

PCB Polychlorobiphenyl

PCR Polimerase chain reaction

PCO Principal coordinates analysis

perMANOVA Permutational analysis of variance

PH Petroleum hydrocarbon

PICRUSt Phylogenetic investigation of communities by reconstruction of unobserved states

QIIME Quantitative insights into microbial ecology

RDP Ribossomal database project

RFLP Restriction fragment length polymorphism

rRNA Ribossomal ribonucleic acid

SBH Southern-blot hybridization

SRB Sulfate reducing bacteria

TC-DNA Total community deoxyribonucleic acid

Thesis Outline

Estuaries are particular environments which are subject to a wide variety of external sources of pressure. The anthropogenic pressure associated to human activities, namely navigation and industries commonly developing around estuarine areas, are often the cause for the rapid degradation of the estuarine environment. Hydrocarbons are a major contaminant in estuarine salt marsh areas and because of the ecological, commercial and economic importance of these wetlands, restoration approaches based on natural recovery processes are being intensively studied.

Microbe-assisted phytoremediation is an inexpensive and environment-friendly approach based on the interactions between plants and their associated indigenous degrading microorganisms (rhizo- and endosphere). Although phytoremediation of a variety of contaminants in different environments has been reported in the last years, studies on phytoremediation of hydrocarbons in salt marsh sediments using halophytes salt marsh plants are strikingly scarce. So, studies focused on the rhizosphere/endosphere microorganisms, essential for salt marsh vegetation to resist environment contamination, could be of great value for the development of new approaches for the mitigation of oil contamination events.

Similarly to what is known for terrestrial plants, the roots of salt marsh halophytes harbour communities of microorganisms that are enriched in important catabolic genotypes for oil hydrocarbon (OH) degradation which may have a potential for detoxification of the surrounding sediment (Geiselbrecht *et al.*, 1996, Daane *et al.*, 2001, Watts *et al.*, 2007). In addition, since rhizosphere bacterial populations may also internalize into plant tissues (endophytes), rhizocompetent OH degrading populations may be important for *in planta* OH degradation and detoxification. In this context, information on how bacterial populations colonizing salt marsh plants respond to oil contamination and how they can contribute to OH detoxification may significantly contribute to the understanding of plant-microbe interactions underlying phytoremediation approaches.

Chapter 1 presents an introduction to the recent scientific knowledge on biodegradation of petroleum hydrocarbons in estuarine environments, the strategies currently available for bioremediation (potential and limitations) and summarizes the

perspectives of the use of halophytes in microbe-assisted phytoremediation approaches. This chapter was published in the journal **Microbial Ecology** with the following reference: MECO-D-14-00127.1; DOI: 10.1007/s00248-014-0455-9

Chapter 2 reports on a thorough in-depth molecular characterization of bacterial communities in bulk sediments and rhizosphere microhabitats of salt marshes of Ria de Aveiro, a temperate estuary chronically exposed to oil hydrocarbon (OH) pollution.
The barcoded pyrosequencing approach used in this study provided a comprehensive overview of the rhizosphere bacterial communities in salt marsh microhabitats [bulk sediment and sediment surrounding the roots (rhizosphere) of *Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*] differentially affected by oil hydrocarbon (OH) pollution. In addition, the 16S rRNA gene sequences retrieved were used to generate *in silico* metagenomes and to evaluate the distribution of potential bacterial traits in different microhabitats. The results revealed that root systems of *H. portulacoides* and *S. perennis* subsp. *perennis* appear to be able to exert a strong influence on bacterial composition. The *in silico* metagenome analyses showed a possible higher number of genes involved in the process of PAH degradation in the rhizosphere of halophyte plants. These observations suggests that halophyte plant colonization can be an important driver of hydrocarbonoclastic bacteria community structure in salt marsh sediments, which can be exploited for in situ phytoremedition of oil hydrocarbon in salt marsh environments. This chapter is submitted for publication in **FEMS Microbiology Ecology** with the following reference: FEM12425; DOI: 10.1111/1574-6941.12425

Chapter 3 reports on a novel insight on hydrocarbon degrading endophytic bacteria of salt marsh plants of a temperate tidal estuary (Ria de Aveiro, Portugal) with the aim to investigate the effects of petroleum hydrocarbon contamination on the structure and function of endophytic bacterial communities of salt marsh plant species (*Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*). Culture-dependent and cultural-independent molecular approaches were used to investigate the effect of petroleum hydrocarbon contamination on the structure and function of endophytic bacterial communities of salt marsh plants. The results demonstrate that the level of

petroleum hydrocarbon contamination affects the composition of endophytic communities, and plant-species-specific “imprints” are confirmed by analyses of PAH (polycyclic aromatic hydrocarbon) degrading genes. These results provide a new insight into the plant-microbe interactions that may ultimately determine the efficiency of microbe-mediated phytoremediation approaches. This chapter was published in the journal **Molecular Ecology** with the following reference: MEC 12559; DOI:10.1111/mec.12559

In **Charter 4**, a plant/sediment microcosm was made to assess if the inoculation of *H. portulacoides* with a hydrocarbon-degrading strain of *Pseudomonas* sp. would mitigate the negative effects of the exposure of the plants to hydrocarbon contamination. 2-methylnaphthalene was used as sediment contaminant source and the halophyte plant *Halimione portulacoides* was chosen as a representative salt marsh plant. A previously isolated PAH-degrading endophytic strain of *Pseudomonas* sp. (chapter 3) was used as plant inoculum. The effect of 2-methylnaphthalene contamination on plant condition was evaluated by measurement of the photosynthetic activity using a non-destructive technique, PAM fluorometry. The responses of microbial communities (rhizosphere and endosphere) in terms of structure [denaturing gradient gel electrophoresis (DGGE)] and function [polycyclic aromatic hydrocarbon (PAH) dioxygenase genes] were assessed. The results indicate that the salt mash plant *H. portulacoides* can influence microbial communities by stimulating the development of hydrocarbon-degrading populations that may contribute to hydrocarbon removal and degradation in salt marsh sediments. The inoculation with an endophytic hydrocarbon-degrading bacterium does not result in a significant effect on the overall hydrocarbon degradation potential of the salt marsh halophyte *H. portulacoides*. However, the effect of the inoculant on the structure of the indigenous endophytic community indicates that the strain may be a good plant colonizer.

Chapter 5 integrates and discusses the main results of this work and attempts to extract the main findings, highlighting conclusions that can be incorporated into innovative and more efficient strategies of microbe-assisted phytoremediation of coastal areas.

Chapter 1

CHAPTER 1

Microbe-assisted phytoremediation of hydrocarbons in estuarine environments

Vanessa Oliveira, Newton C.M. Gomes, Adelaide Almeida, Artur M. S. Silva, Helena Silva & Ângela Cunha

Accepted in Microbial Ecology journal

Abstract: Estuaries are sinks for various anthropogenic contaminants, such as petroleum hydrocarbons, giving rise to significant environmental concern. The demand for organisms and processes capable of degrading pollutants in a clean, effective, and less expensive process is of great importance. Phytoremediation approaches involving plant/bacteria interactions have been explored as an alternative, and halophyte vegetation has potential for use in phytoremediation of hydrocarbon contamination. Studies with plant species potentially suitable for microbe-assisted phytoremediation are widely represented in the scientific literature. However, the in-depth understanding of the biological processes associated with the re-introduction of indigenous bacteria and plants and their performance in the degradation of hydrocarbons is still the limiting step for the application of these bioremediation solutions in a field context.

The intent of the present review is to summarize the sources and effects of hydrocarbon contamination in estuarine environments, the strategies currently available for bioremediation (potential and limitations), and the perspectives of the use of halophyte plants in microbe-assisted phytoremediation approaches.

Keywords: salt-marshes; hydrocarbons; halophytes; phytoremediation

Introduction

Salt marshes are complex coastal environments usually situated within estuarine systems. They represent dynamic habitats, developing along the coast line and inside estuaries and are characterized by high concentration of soluble salts (prevailing NaCl), relatively low diversity of species and high biomass productivity (Vernberg, 1993, Baptista *et al.*, 2011). Estuarine salt marshes are among the most productive ecosystems on Earth (Costanza *et al.*, 1997) promoting plant and microbial activity (Cunha *et al.*,

2005, Santos *et al.*, 2007, Oliveira *et al.*, 2010), representing a preferential habitat for many organisms (fish, bird and other wildlife) (Mitsch & Gosselink, 2000, Watts *et al.*, 2006) and providing important ecosystem services (Barbier *et al.*, 2011). Salt marshes are highly dynamic areas, influenced by the joint action of water, sediment, and vegetation, providing a buffer zone between terrestrial and aquatic ecosystems in urban and industrial areas. They contribute to flood control and erosion prevention and may act as protective filters and final repositories for runoff pollutants, pathogens and nutrients (Vernberg, 1993, Valiela *et al.*, 2004, Barbier *et al.*, 2011).

Salt marshes are sinks for various pollutants, receiving important anthropogenic inputs from urban areas, industries and agricultural compounds, namely polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), hexachlorocyclohexane (HCH) and hexachlorodimethanonaphthalene (Dieldrin), as summarized in Table 1.1. Due to their ecological importance, the cleanup and recovery of these ecosystems is an issue of public concern.

Petroleum hydrocarbons (PHs) represent one of the most common groups of persistent organic pollutants in coastal and estuarine systems (Mucha *et al.*, 2011). They are continuously released, persistent in the environment, toxic to many organisms, and hazardous to human health (Todd *et al.*, 1999). This class of contaminants may be originated from industrial release products or from accidental spills (Todd *et al.*, 1999). Numerous studies indicate that salt marsh sediments are capable of retaining PHs and that the stimulation of microbial activity in the rhizosphere of plants can accelerate their biodegradation (Carman *et al.*, 1996, Daane *et al.*, 2001). Phytoremediation is one of the processes of hydrocarbon bioremediation, which has been intensively studied in the last decade. The continuous release of hydrocarbons and their degradation products caused by anthropogenic activities around estuary areas leads to the necessity for efficient, inexpensive, and environmental friendly processes of hydrocarbon decontamination, such as phytoremediation. In that perspective, the interactions between halophytes, plants capable of growing in salt marshes, and their root-associated bacteria may play a relevant role in the remediation of contaminated areas. Cultivation-dependent and -independent approaches together with molecular approaches have been used to characterize plant hydrocarbonclastic bacteria partnerships in the perspective of their

exploitation for microbe-assisted phytoremediation. However, particular features of salt marsh ecosystems may impose difficulties in the process of implementation of these strategies in the field, and considerable research effort has been directed to a deeper understanding of halophyte-microbe interactions in oil-polluted environments.

The purpose of this paper is to summarize recent knowledge on the degradation of petroleum hydrocarbons in salt marsh sediments and to critically discuss the potential and limitations of microbe-assisted phytoremediation approaches for the recovery of oil-impacted ecosystems.

Table 1.1 Concentration of various pollutants detected in sediments at estuaries from around the world.

Site	Pollutant[a]	Concentration (µg/g)[b]	Reference
Charleston Harbor Estuary, South Carolina, USA	Al	8.54	Sanger *et al.*, 1999
	Cd	0.28	
	Cr	94.1	
	Cu	32.4	
	Fe	4.28	
	Pb	31.7	
	Mn	247.7	
	Hg	0.13	
	Ni	28.1	
	Zn	111.7	
Chesapeake Bay, Maryland, USA	Cd	17.6	McGee *et al.*, 1999
	Cr	1,831.1	
	Cu	396	
	Fe	14.74	
	Mn	3,381.0	
	Ni	157.7	
	Pb	348.6	
	Zn	2,105.4	
	Hg	2,340.3	
	PAHs	23,322.8	
	PCBs	2,148.2	
San Francisco Bay, California, USA	Cd	5.733	O'Day *et al.*, 2000
	Ni	39.677	
	Cu	126.076	
	Pb	265.229	
	Zn	295.527	

Table 1.1 (continued)

Site	Pollutant[a]	Concentration (µg/g)[b]	Reference
Humber Estuary, Eastern England	Cu	60	Lee & Cundy, 2001
	Pb	127	
	Zn	344	
	HCB	0.022	
	HCH	0.003	
	Dieldrin	0.167	
Suir Estuary, Ireland	Cu	23.194	Fitzgerald *et al.*, 2003
	Pb	69.208	
Bay of Fundy, Canada	Hg	0.079	Hung & Chmura, 2006
Mersey Estuary, U.K.	PAHs	3.766	Vane *et al.*, 2007
	PCBs	1.409	
Salt marsh along coastal zone of Portugal	Hg	> 10 to < 0.5 ppm	Valega *et al.*, 2008
Mitrena salt marsh, Sado, Portugal	PAHs	7.35	Martins *et al.*, 2008
Yangtze River intertidal zone, China	Al	97213	Zhang *et al.*, 2009
	Fe	49627	
	Cd	0.750	
	Cr	173	
	Cu	49.7	
	Mn	1112	
	Ni	48	
	Pb	44.1	
	Zn	154	
Cávado River estuary, Portugal	PAHs	0.4023	Mucha *et al.*, 2011
Lima River estuary, Portugal	PAHs	800	Ribeiro *et al.*, 2011

[a] PAHs: polycyclic aromatic hydrocarbons; PCBs: polychlorobiphenyls; DDT: dichlorodiphenyltrichloroethane; HCB: hexachlorobenzene; HCH: hexachlorocyclohexane; Dieldrin:hexachlorodimethanonaphthalene.
[b] Maximum concentration found in soil or sediments.

Hydrocarbons

Petroleum hydrocarbons (PHs) are common environmental contaminants and represent a serious problem in many parts of the world (Gioia *et al.*, 2006, Iqbal *et al.*, 2007, Labbe *et al.*, 2007, Vazquez *et al.*, 2009, Yergeau *et al.*, 2009, Santos *et al.*, 2011), particularly in coastal and estuarine systems which may become seriously affected (Castle *et al.*, 2006, Gomes *et al.*, 2008, Beazley *et al.*, 2012). They are the principal components in a range of commercial products (e.g., gasoline, fuel oils,

lubricating oils, solvents, mineral spirits, mineral oils, and crude oil). Petroleum products are a complex mixture of hundreds of hydrocarbon compounds, including various amounts of aliphatic and aromatic molecules. They enter and spread through the environment in many different ways (Todd *et al.*, 1999). Certain petroleum hydrocarbons are directly released in the water column, forming surface films while others tend to accumulate in the sediment.

PAHs are widespread in air, soil, sediment, surface water, groundwater, and runoff and are also found to accumulate in plants and aquatic organisms (Simpson *et al.*, 1996, Peng *et al.*, 2008, Haritash & Kaushik, 2009). In estuarine environments, polycyclic aromatic hydrocarbons (PAHs) are of great concern due to their potential for bioaccumulation, persistence, transport, toxicity, mutagenicity and carcinogenicity (Cerniglia, 1992, Samanta *et al.*, 2002, Bamforth & Singleton, 2005, Haritash & Kaushik, 2009). These compounds are introduced into estuarine environments from different sources and by a variety of processes (Fig. 1.1). Although PAHs are ubiquitous in the environment (fossil fuels, brush fires, volcanoes, and burning natural vegetation), anthropogenic activities, such as petroleum refining and transport activities dependent on the combustion of fossil fuels, are the major contributors to their release in the environment (Freeman & Cattell, 1990, Cerniglia, 1992, Samanta *et al.*, 2002, Bamforth & Singleton, 2005).

PAHs are classified as low molecular weight (LMW) and high molecular weight (HMW) according to the number and type of rings they have in the structure (Bayoumi, 2009). Based on their abundance and toxicity, 16 PAHs have been included in the list of priority pollutants of the US Soil Protection Agency (Liu *et al.*, 2001). Because of their high hydrophobicity and low lability, the process of PAHs remediation, especially in soils and sediments, is generally slow and expensive. The fate of PAHs in the environment depends on abiotic and biotic processes such as stabilization, landfarming (stimulation of indigenous microorganisms in the soil by providing nutrients, water, and oxygen), steam and thermal heating, chemical oxidation, bioremediation (bioaugmentation and biostimulation), and phytoremediation, which have been applied to the restoration of groundwater and soils/sediments (Bamforth & Singleton, 2005, Wick *et al.*, 2011).

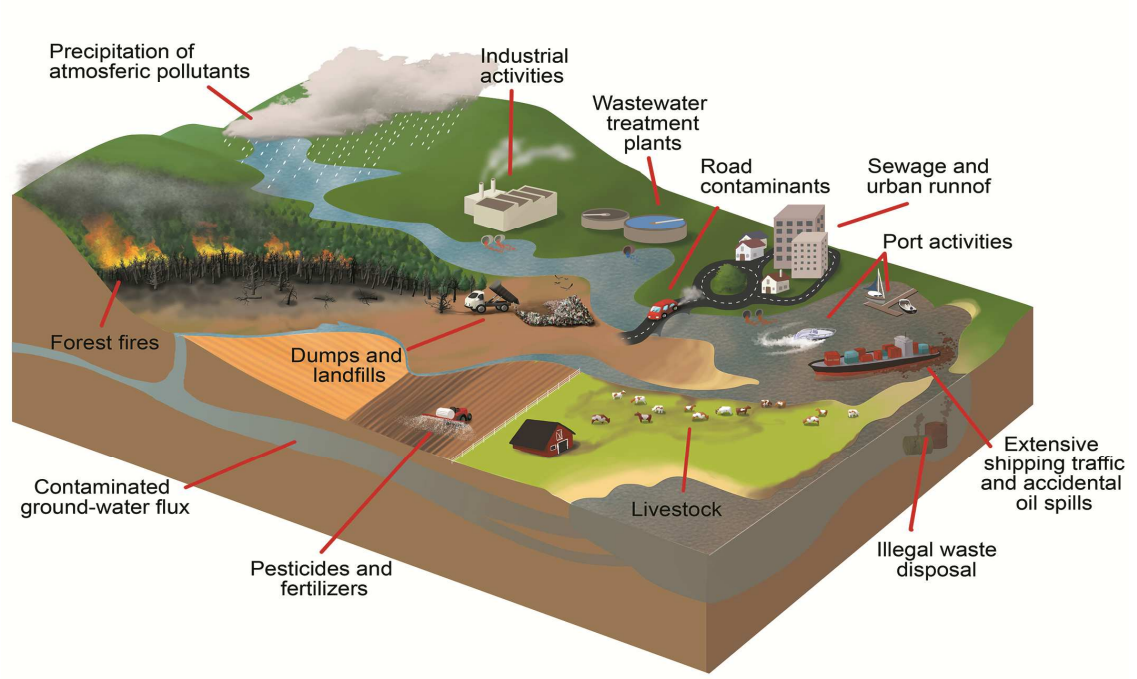

Fig. 1.1 Sources and processes involved in hydrocarbon release into estuarine ecosystems.

Phytoremediation of hydrocarbons

Halophytes are defined as plants capable of completing their life cycle in salt concentrations around 0.200M NaCl or even higher (Flowers & Colmer, 2008). Moreover, many of these plants inhabit environments subject to constant flooding (e.g coastal mangroves and salt marshes) (Colmer & Flowers, 2008). Because estuaries and coastal habitats are highly exposed to environmental contamination, many studies addressed the use of halophytes in the phytoremediation of many pollutants (e.g., heavy metals, xenobiotics, and PHs) (Lin & Mendelssohn, 2009, Couto *et al.*, 2011, Liu *et al.*, 2011, Marques *et al.*, 2011, Curado *et al.*, 2014). The physiological mechanisms that these plants use to tolerate salts are partly analogous to those involved in heavy metal resistance. Therefore, halophyte plants can accumulate metals, being therefore regarded as promising candidates for the removal or stabilization of heavy metals in polluted soils (Manousaki & Kalogerakis, 2011).

According to the fate of the contaminant or to the mechanism by which plants remediate contamination, these approaches are referred to as phytoextraction, rhizofiltration, phytostabilization, phytovolatilization, phytodegradation or

rhizodegradation (EPA, 2000, Trapp & Karlson, 2001). Phytoextraction refers to the uptake, translocation, and accumulation of contaminants in the soil by plant roots into above-ground components of the plants. This technique involves the introduction of plants referred to as hyper-accumulators in polluted sites that after grown, are harvested. So, phytoextraction involves the repeated cropping of plants in contaminated soil until contaminant concentration decreases to acceptable levels. After harvesting, contaminated biomass needs treatments prior to disposal that can pass to secure landfills, incineration, or more recently thermo-chemical conversion processes (combustion, gasification, and pyrolysis) (Garbisu & Alkorta, 2001). Rhizofiltration involves the absorption or adsorption of contaminants through roots or other plant parts (EPA, 2000, Trapp & Karlson, 2001). In phytostabilization, plants reduce the bioavailability of contaminants immobilizing them in soil/sediment, reducing the mobility of contaminants and preventing migration to water or air (EPA, 2000, Trapp & Karlson, 2001). For the removal of low molecular weight compounds from soil, phytovolatilization is used. In this technique, plants volatilize contaminants that are biologically converting to gaseous species and releasing them through leaves via evapotranspiration processes (EPA, 2000, Trapp & Karlson, 2001). Organic contaminants such as petroleum, PAHs, BTEX, TNT, chlorinated solvents, and pesticides are degraded only by plants (phytodegradation) or by microorganisms and plants, in a process denominated as rhizodegradation (Trapp & Karlson, 2001).

Comparatively with application for the sequestration or removal of metals, studies involving halophytes for phytoremediation of PHs are still rather scarce. However, species of *Spartina*, *Salicornia*, *Juncus*, *Halonemum*, *Halimione*, and *Scirpus* have been tested for the remediation of hydrocarbons in wetlands with encouraging results (Table 2). The ability for PAH bioaccumulation was described for *Salicornia fragilis* shoots by a process of soil-to-plant transference that is dependent of exposure duration and pollution degree. High molecular weight PAHs were detected in aerial parts of the plant (Meudec *et al.*, 2006). In a study conducted in greenhouse conditions, the use of *Juncus roemerianus* transplanted to salt marsh sediment contaminated with different diesel oil dosages was tested. The results revealed the reduction of PHs in *J. roemerianus* treatments, in relation to control sediments, suggesting that these plants may simultaneously contribute to the restoration and remediation of diesel-contaminated

wetlands. Phytoremediation by *J. roemerianus* was even more effective for PAHs than for *n*-alkanes (Lin & Mendelssohn, 2009). In an outdoor laboratory experiment (microcosm-scale), the potential of the salt marsh plants *Halimione portulacoides*, *Scirpus maritimus*, and *Juncus maritimus* for the remediation of soil contaminated with refinery waste was tested. Two situations were tested: (i) the use of each individual plant species or the use of an association of two plants (*S. maritimus* and *J. maritimus*) and (ii) soil with old contamination (crude oil) or a mixture of the old and recent (turbine oil) contamination. Combined transplants of *S. maritimus* and *H. portulacoides* plants were efficient in removing not only all the recent and old contamination, and the process was faster and more efficient than natural attenuation (Couto *et al.*, 2011).

Table 1.2 Halophytes used for phytoremediation of hydrocarbons in estuarine areas.

Plant	Result	Reference
Spartina alterniflora and *Spartina patens*	Restoration of oil contaminated wetlands and accelerated oil degradation in soil.	Lin & Mendelssohn, 1998
Salicornia fragilis	Intense bioaccumulation of PAHs from oil-polluted sediments in the shoots.	Meudec *et al.*, 2006
Juncus roemerianus	Phytoremediation of diesel-contaminated wetlands.	Lin & Mendelssohn, 2009
Halonemum strobilaceum	Phytoremediation of oil-polluted hypersaline environments via rhizosphere technology.	Al-Mailem *et al.*, 2010
Halimione portulacoides, *Scirpus maritimus* and *Juncus maritimus*	Removal of petroleum hydrocarbons from soil.	Couto *et al.*, 2011
Scirpus triqueter	Enhanced biodegradation of diesel pollutants.	Liu *et al.*, 2011

Despite existing evidence that the halophytes can be used successfully for the phytoremediation of estuarine areas, such as oil- or diesel-polluted sites (Table 1.2), there are still some limitations to the extensive use of this bioremediation approach. The efficiency of halophytes, as phytoremediation agents, depends on the plant species. For example, a comparative study of the efficiency of the salt-marsh species *H. portulacoides*, *S. maritimus*, and *J. maritimus* for remediation of PHs revealed that the plant species is determinant in the rate of hydrocarbon clearance and, more importantly,

that different associations between these plants can interfere with or even inhibit the process (Couto *et al.*, 2011).

Microbial hydrocarbon degradation

Contrasting with the prospective character of the use halophytes, the use of microorganisms for the clearance of hydrocarbons in the environment has long been regarded with interest. Through microbial activity, hydrocarbons are converted into carbon dioxide, water, and living biomass (Genouw *et al.*, 1994). A diversity of bacteria, fungi and algae has been characterized as to their capacity to degrade PAHs (Haritash & Kaushik, 2009).

Microorganisms have been found to degrade PAHs via different catabolic pathways, such as anaerobic or aerobic metabolism, or co-metabolism which is important for the degradation of mixtures of PAHs and high molecular weight PAHs (Habe & Omori, 2003, Zhong *et al.*, 2007, Peng *et al.*, 2008). The initial step in aerobic metabolism of PAHs usually occurs via the incorporation of oxygen into aromatic rings followed by the systematic breakdown of the compound to PAHs metabolites and/or carbon dioxide. Anaerobic metabolism occurs via hydrogenation of aromatic rings (Bamforth & Singleton, 2005, Zhong *et al.*, 2007). In co-metabolism, the range and extent of high molecular weight PAH degradation is influenced by an important interaction that transforms the non-growth substrate (PAHs) in the presence of growth substrates (Zhong *et al.*, 2007).

Physicochemical factors, such as soil type and structure, pH, temperature, electron acceptors, and nutrients, will affect microbial activity and determine the persistence of hydrocarbons (such PAHs) in polluted environments (Table 1.3). In estuarine areas, such as salt marshes, salinity fluctuations represent one of major challenges for hydrocarbon degradation that may even compromise the overall success of the process. There is an inverse relation between salinity and hydrocarbon solubility, with the consequent inhibitory effect of salinity on hydrocarbon bioremediation (Mille *et al.*, 1991). However, successful hydrocarbon degradation has been reported over a wide range of salinity values. A consortium of bacteria isolated from oil-contaminated sediments demonstrated the highest rate of hydrocarbon degradation with a salinity of 0.4M NaCl and the degradation was attenuated below and above this limit (Bertrand *et*

al., 1993). In a study with two bacterial consortia isolated from crude oil and mangrove sediments, the highest rate of degradation of aliphatic and aromatic hydrocarbons occurred in a salinity range between 0-0.171M, and decreased with increasing salinity (Beeson *et al.*, 2002).

Table 1.3 Factors affecting the biodegradation of petroleum hydrocarbons**.**

Factor	Effect	Reference
Bioavailability	Composition and concentration of hydrocarbons affect the rate and extent of biodegradation.	Venosa & Zhu, 2003, Peng *et al.*, 2008, Das & Chandran, 2010
Temperature	Affects the physicochemical behavior of hydrocarbons (viscosity, diffusion, solubility).	Venosa & Zhu, 2003, Coulon *et al.*, 2007, Peng *et al.*, 2008, Das & Chandran, 2010
	Affects the physiology and diversity of microorganisms.	
pH	Inhibits microbial activity by regulating microbial metabolism.	Venosa & Zhu, 2003, Aislabie *et al.*, 2006, Peng *et al.*, 2008)
Nutrients	Availability of limiting nutrients (N, P, K, Fe) affects microbial growth and consequently biodegradation rates.	Venosa & Zhu, 2003, Kaakinen *et al.*, 2007, Peng *et al.*, 2008, Das & Chandran, 2010
Oxygen	Despite the occurrence of biodegradation of hydrocarbons in anaerobic and aerobic conditions, oxygen depletion decreases biodegradation rates.	Venosa & Zhu, 2003, Peng *et al.*, 2008, Haritash & Kaushik, 2009
Salinity	Changes in salinity promote alteration of the microbial population that affects biodegradation rates. High concentration of salt inhibits hydrocarbon degradation.	Venosa & Zhu, 2003, Minai-Tehrani *et al.*, 2009
Organic matter	Promotes sequestration of contaminants, interfering in their availability.	Banks *et al.*, 2003, Haritash & Kaushik, 2009, Afzal *et al.*, 2011
Soil type and structure	Soil type influences the bacterial colonization and microbial activities and subsequently the efficiency of contaminant degradation.	Radwan *et al.*, 2005, Afzal *et al.*, 2011

Riis *et al.*, (2003) reported diesel fuel degradation by microbial communities from saline soils in Patagonia up to a salinity of 2.997M. Although hydrocarbon contamination is still persistent and recalcitrant in its nature, the fact that microbes from vegetated saline sediments can still actively degrade hydrocarbons in the presence of variable and relatively high concentrations of salt opens promising perspectives for microbe-assisted phytoremediation in estuarine areas.

Microbe-assisted phytoremediation

The microbial communities associated with plants and plant-microbe interactions established between them, have a significant role in the physiology and health of the plant, exerted through inhibition of phytopathogens (e.g., antibiotic and siderophore production or nutrient competition), release of growth-promoting molecules, enhancement of nutrient availability, promotion of detoxification (e.g., sequestration, volatilization, and degradation of pollutants), and improvement of stress tolerance by induction of systematic acquired host resistance (Fig. 1.2).

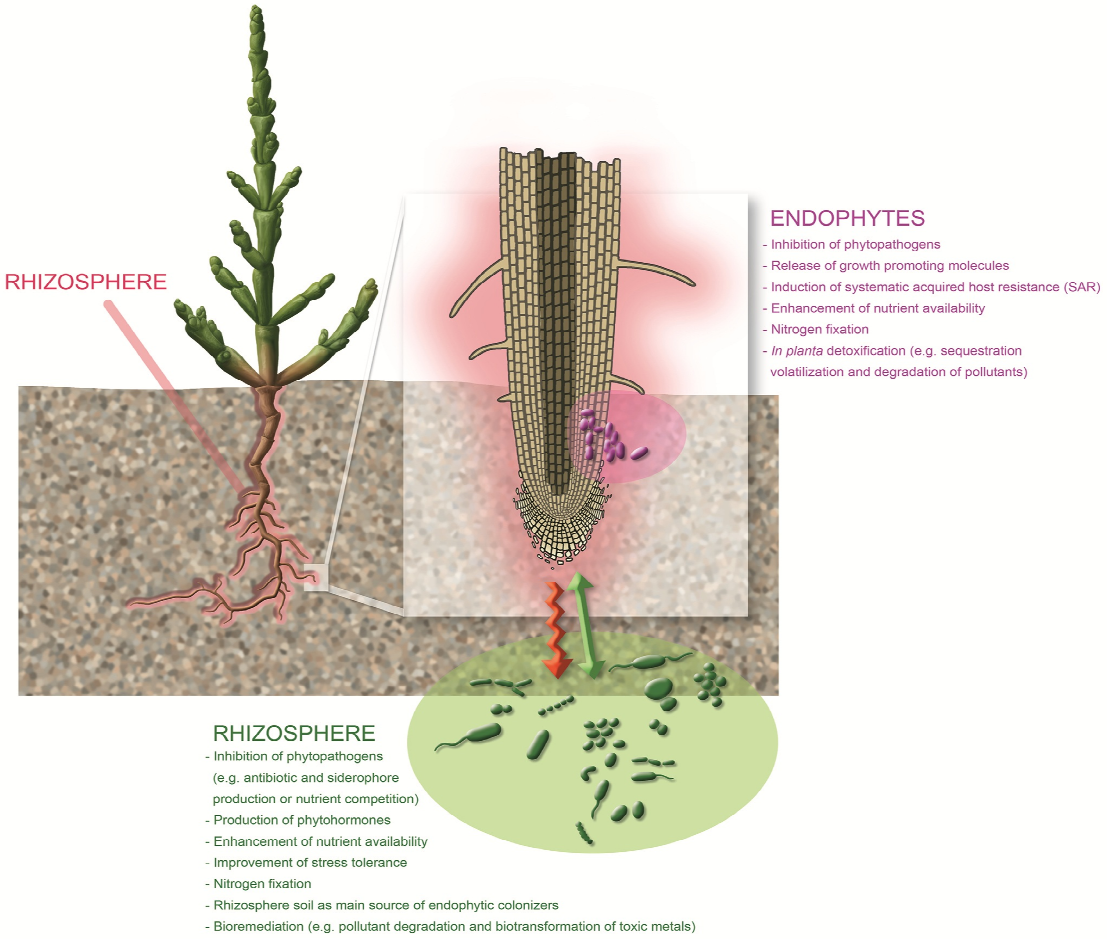

Fig. 1.2 Plant-microbe interactions and plant-growth-promoting effects of rhizosphere and endosphere bacteria.

The use of plants and their associated microorganisms for the removal of contaminants from the environment is based on the increase of microbial population numbers in the rhizosphere and/or endosphere and on the stimulation of their metabolic

activity (Kuiper *et al.*, 2004). So, microbe-assisted phytoremediation represents a powerful emerging approach to sequester, degrade, transform, assimilate, metabolize, or detoxify contaminants from soil, sediment, or groundwater (EPA, 2000, Moreira *et al.*, 2006).

Numerous bacteria found in association with plants are capable of degrading hydrocarbons, namely PAHs, suggesting that indigenous rhizobacteria and endophytic bacteria may have potential for bioremediation of polluted sites (Segura *et al.*, 2009). In the particular case of estuarine environments, a diversity of hydrocarbon-degrading microbial populations has been found in association with the rhizosphere of salt marsh plants, where they actively contribute to hydrocarbon removal and degradation (Daane *et al.*, 2001, Ribeiro *et al.*, 2011). The use of rhizosphere and phyllosphere (aerial portion of plants) of the halophyte *Halonemum strobilaceum* was also proposed for phytoremediation of oil-polluted hypersaline environments, via rhizosphere technology (Al-Mailem *et al.*, 2010).

Rhizodegradation appears to be a particularly interesting phytoremediation process for the removal and/or degradation of organic contaminants, such as PH. The rhizosphere is defined as the zone directly influenced by the plant root system. Plants provide root exudates rich in carbon sources, nutrients, enzymes, and sometimes oxygen, creating a favorable environment in which microbial activity is stimulated (Cunningham *et al.*, 1996, Frick *et al.*, 1999, Kuiper *et al.*, 2004). However, microbial interactions with plants are not limited to the rhizosphere; rather, they extend to the interior of the plant (Lodewyckx *et al.*, 2002). Endophytic-assisted phytoremediation, involving microorganisms that are capable of living within various plant tissues (roots, stems, and leaves), has been reported in recent years as successful in the degradation of some pollutants, such as explosives, herbicides, and hydrocarbons (Germaine *et al.*, 2006, Phillips *et al.*, 2008, Segura *et al.*, 2009). In fact remediation of hydrocarbons by combined use of plants and rhizobacteria and/or endophytic bacteria have been widely described (Table 1.4).

Genetically engineered endophyte microorganisms enhance the overall health of theirs hosts (Barac *et al.*, 2004) and may indirectly improve biodegradation of contaminants in the rhizosphere. Experiments in which pea plants were inoculated with the naphthalene degrader *Pseudomonas putida* VM1441 (pNAH7) and exposed to

naphthalene contamination revealed that naphthalene degradation rate (~ 40%), seed germination, and plant transpiration were enhanced in inoculated plants than in non-inoculated controls. Moreover, inoculation resulted in an overall protection of the host plants from the phytotoxic effects of naphthalene (Germaine *et al.*, 2009). A study carried out in a mangrove showed that nursery conditions and early microbial colonization patterns had long-term effect on the rhizosphere of transplanted mangroves. This phenomenon may have potential application for introducing new rhizocompetent bacteria carrying genes or plasmids to improve plant growth or bioremediation (rhizoengineering) (Gomes *et al.*, 2010b).

Several studies reported the enhancement of PH degradation in association with the presence of bacteria carrying PH degradation genes (Table 1.4). A high diversity of hydrocarbon degradative genes, such as alkane monooxygenase (*alkB*), naphthalene dioxygenase (*ndoB*), phenanthrene dioxygenase (*phnAc*), and cytochrome P450 alkane hydroxylase, has been detected in plant microhabitats (rhizosphere and endosphere) (Phillips *et al.*, 2006, Phillips *et al.*, 2008, Germaine *et al.*, 2009). In fact, the monitoring of gene abundance and expression during phytoremedition of contaminated sites can give indications about the persistence and functional activity of inoculated microorganisms (Juhanson *et al.*, 2009). A study conducted at a long-term phytoremediation field site revealed that both rhizosphere and endophytic communities showed substantial inter-species variation in hydrocarbon degradation potential and activity levels, with an increase in catabolic genotypes in specific plant treatments (Phillips *et al.*, 2008).

Recently, it was suggested that for certain phytoremedition approaches, it may be essential or at least important, that bacteria also act as plant growth promoters, in addition to their pollutant-degrading activity. In experiments with Italian ryegrass, plant biomass production and alkane degradation were significantly enhanced by inoculation with bacterial strains expressing hydrocarbon-degrading genes (e.g. *alkB*) as well as plant-promoting activity (1-aminocyclopropane-1-carboxylate (ACC) deaminase activity) (Yousaf *et al.*, 2011, Afzal *et al.*, 2012). So, the combined use of plant and bacteria can be exploited to relieve plant stress, and enhance bioremediation of PH-contaminated sites.

Table 1.4 Examples of successful approaches of remediation of hydrocarbons by combined use of plants and rhizobacteria and/or endophytic bacteria.

Rhizobacteria or endophytic bacteria	Plant	Gene(s)[a]	Plant-growth promotion features[b]	Type of contaminant	Percen. Degradation[c]	Reference
Pseudomonas sp. GF3	*Triticum aestivum*	unknown phenanthrene degradation gene		Phenanthrene	84.8% in 80 days	Sheng & Gong, 2006
Culturable n-hexadecane degraders	*Festuca rubra*	*alkB*, *ndoB*, *nidA*, *phnA*c and C2,3O		Mixture of hydrocarbons[d]	50% in 4.5 months	Phillips *et al.*, 2006
Pseudomonas putida VM1441(pNAH7)	*Lolium multiflorum*	*nah* (plasmid NAH7)		Naphthalene	40% in 14 days	Germaine *et al.*, 2009
Pseudomonas strains, UW3 and UW4	*Lolium perenne*, *Festuca arundinacea*, and *Secale cereale*		ACC deaminase, siderophores and IAA producing strain	Oil refinery	65% in 3 years	Gurska *et al.*, 2009
Azospirillum brasilense SR80	*Secale cereale* and *Medicago sativa*		IAA producing strain	Oil sludge	approx. 70 % in 120 days	Muratova *et al.*, 2010
Pantoea sp. strains, ITSI10 and BTRH79; *Pseudomonas* sp. strains, ITRI15 and ITRH76	*Lolium multiflorum* and *Lolium corniculatu*s	unknown alkane degradation gene; cytochrome P450 alkane hydroxylase and *alkB* gene		Diesel fuel	> 57% in 90 days	Yousaf *et al.*, 2010a
Gordonia sp. S2RP-17	*Zea mays*		ACC deaminase and siderophores	Diesel fuel	96 % in 46 days	Hong *et al.*, 2011
Pantoea sp. strains, ITSI10 and BTRH79; *Pseudomonas* sp. MixRI75	*Lolium multiflorum*	unknown alkane degradation gene; cytochrome P450 alkane hydroxylase and *alkB* gene	ACC deaminase producing strain	Diesel fuel	approx. 79 % in 93 days	Afzal *et al.*, 2012
Pseudomonas sp. SB	*Testuca arundinacea*		ACC deaminase, siderophores and IAA producing strain	Oil	85% in 120 days	Liu *et al.*, 2013

[a] *alkB*: alkane monooxygenase; , *ndoB*: naphthalene dioxygenase; *nidA*: naphthalene inducible dioxygenase; *phnAc:* phenanthrene dioxygenase; C2,3O: catechol 2,3 dioxygenase; nah: encoded same proteins for both upper and lower pathway of naphthalene degradation.
[b] ACC: 1-aminocyclopropane-1-carboxylate deaminase activity; IAA: indole-3-acetic acid.
[c] Maximum degradation obtain ed from sediments or soils in each study.
[d] Hydrocarbon contaminated site located in south-eastern Saskatchewan, Canada (approx. 3000 to 3500 ug/g).

Current limitations to the microbe-assisted phytoremediation of hydrocarbons

Microbe-assisted phytoremediation has been broadly tested for the degradation or sequestration of hydrocarbons in estuarine environments. Despite being considered an inexpensive, sustainable and environment-friendly technique, phytoremediation is not exempt of controversy, and the success of this type of approach is significantly affected by environmental factors and particular features of each ecosystem. One major limitation is time, considering that successful phytoremediation is a process that goes on for long periods which is partially determined by the slow growth and phenological (or life) cycle of plants, the limited depth of the root system, and the fact that many plant species are sensitive to the contaminants that are being remediated (Kuiper *et al.*, 2004, Pilon-Smits, 2005).

The bioavailability of petroleum hydrocarbons is another important factor in the success of bioremediation, and it can be significantly affected by soil type and organic matter content. Water content (affects the availability of oxygen required for aerobic respiration), temperature, and nutrient availability (influences the rate and extent of biodegradation) are relevant determinants of the efficiency of the PHs bioremediation process (Frick *et al.*, 1999). The competition for nutrients between plants and microorganisms can be a restriction to the remediation efficiency. A reduction in microbial abundance and an attenuation of degradation of higher molecular weight PAHs in sediments was observed in *H. portulacoides* banks and this effect was associated to nutrient limitation (Mucha *et al.*, 2011). Therefore, fertilization may be required for optimal rhizoremediation of hydrocarbons.

Although a wide range of hydrocarbon-degrading bacteria have been isolated from contaminated environments, little is known about the stability of the association with salt marsh plants and the success of the re-introduction of plant-bacteria systems for potential phytoremediation processes in saline sediments. The fact that these biotopes are colonized in a particular type of plants, well adapted to flooding and to salinity fluctuations, reinforces the need to incorporate basic knowledge on their interaction with sediment microbes in the design of phytoremediation approaches. The ability to monitor the survival and efficiency of hydrocarbon degradation of inoculated strains is essential for the in-depth understanding of the network of relations established between sediments, plants, and microbes that underlies microbe-assisted phytoremediation.

Monitoring plant-bacteria interactions involved in microbe-assisted phytoremediation

The efficient colonization of plants by microbial pollutant degraders is an essential contribution for plant survival and hydrocarbon degradation (Andria *et al.*, 2009, Afzal *et al.*, 2012). Despite the lack of knowledge on inoculation and bacterial colonization of halophyte plants, the monitoring of plant-bacteria interaction in hydrocarbon-polluted sites has been addressed by different approaches. In bioremediation, the use of culture-dependent methodologies, such as dilution plating on agar plates containing antibiotics, the most-probable-number (MPN) method, and direct counting, are insufficient for an accurate and sensitive monitoring of the inoculation and colonization processes (Schneegurt & Kulpa, 1998). Molecular techniques including polymerase chain reaction (PCR), real-time PCR, and DNA hybridization, reporter genes or genetically marker microorganisms (biomarkers) have been used to check on microbe survival, efficiency of colonization, and activity (Germaine *et al.*, 2004, Ryan *et al.*, 2008, Andria *et al.*, 2009, Juhanson *et al.*, 2009, Segura *et al.*, 2009). For example, antibiotic resistance and green fluorescent protein (*gfp*) genes have been proposed as useful tools for monitoring the colonization of bacterial endophytes, inoculated in poplar trees (Afzal *et al.*, 2013). Endophyte colonization has also been monitored with the use of *gus*A marker gene encoding the enzyme β-glucuronidase. A *gus*-marked strain, *Burkholderia phytofirmans* PsJN, was inoculated in seeds of ryegrass (*Lolium multiflorum* Lam.). *B. phytofirmans* PsJN:gusA10 revealed that this bacterium has the ability to colonize the rhizosphere and endosphere of ryegrass vegetation in a diesel-contaminated soil and generally improved plant biomass production and hydrocarbon degradation (Afzal *et al.*, 2013). Other study, using restriction fragment length polymorphism (RFLP), showed that *Enterobacter ludwigii* strains were able to efficiently colonize the rhizosphere and endosphere of Italian ryegrass, birdsfoot trefoil, and alfalfa. Moreover, *E. ludwigii* strains contain a cytochrome P450-type alkane hydroxylase (CYP153), and the quantification and expression of these genes by real-time PCR indicate an active role in hydrocarbon degradation, in the rhizosphere and endosphere of all three plant species (Yousaf *et al.*, 2011). Quantitative PCR has emerged as a useful and rapid tool for monitoring catabolic genes during bioremediation processes. As an example, this technique was used for the assessment of hydrocarbon degradation activity of *Nocardia sp. H17-1*

during remediation of crude-contaminated soil (Baek *et al.*, 2009). A similar approach was used to demonstrate that hydrocarbon degradation was associated with functional changes in microbial communities, in which high copy numbers of catechol 2,3-dioxygenase and naphthalene dioxygenase correlated with PAH mineralization (Phillips *et al.*, 2012).

Metagenomic pyrosequencing, which allows the recovery of a very large number of microbial sequences directly from environmental samples, has more recently emerged as a powerful technique to follow plant-microbe interactions during the bioremediation process (Roesch *et al.*, 2007, Wang *et al.*, 2012). The sequences obtained can be compared with reference libraries, and then taxa present in an environmental sample can be identified with high confidence. The massive data sets generated provide information that can be used for a variety of applications, such as the comprehensive understanding of within-site and between-site variability of microbial communities and the impact of this variability in ecosystem-scale processes in salt marshes (Bowen *et al.*, 2012). The pyrosequencing analysis of bacterial 16S ribosomal RNA (16S rRNA) gene fragments of different *Phragmites australis* rhizospheres revealed a trend in the variation of bacterial community structure during wetland degradation and identified sulfur and sulfate-reducing bacteria, nitrifying and nitrogen-fixing bacteria, and methane-oxidizing bacteria as crucial in the protection and ecological restoration of wetlands (Zhang *et al.*, 2013). Recent bioremediation studies have used pyrosequencing analysis of bacterial 16S rRNA genes to describe microbial community dynamics in hydrocarbon-contaminated sites thus providing basis for the development of strategies for monitoring remediation processes (Singleton *et al.*, 2011, Sutton *et al.*, 2013). For example, the relative abundance of *Chloroflexi*, *Firmicutes*, and *Euryarchaeota* was directly correlated with the presence of diesel (Sutton *et al.*, 2013).

Future perspectives

Despite numerous limitations, phytoremediation and particularly, microbe-assisted phytoremediation have undeniable advantages, and research must now specifically address the aspects that can allow the scaling up from laboratory to the field for the practical implementation of this approach.

Each salt marsh displays particularly biological, chemical, and physical characteristics that will ultimately determine the success of phytoremediation. Therefore, field studies, combined with laboratory approaches, are required for the understanding of the interplay of biological and chemical processes involved in microbe-assisted phytoremediation of oil-impacted sites.

Considering that plant-microbe interactions play a key role in the process of environment and *in planta* detoxification, (a) the identification of autochthonous hydrocarbon-degrading bacterial populations associated to salt marsh plants (rhizosphere and aboveground plant tissues), (b) the identification of degradative plasmids, and (c) the selection of petroleum-resistant plants are key issues for the success of environmental restoration. The detection of genes related to hydrocarbon degradation pathways in halophyte plants can be useful to screen for lineages of plants that can be used in efficient phytoremediation protocols. Moreover, these genes can be used for the genetic engineered design of plants for novel phytoremediation approaches for hydrocarbon-polluted wetlands and soils (RamanaRao *et al.*, 2012). Recent plant biotechnology approaches involving the introduction of specialized bacterial endophytes in plants or the design of genetically engineered plants containing interesting bacterial genes (Barac *et al.*, 2004, Taghavi *et al.*, 2005) create new perspectives for future phytoremedition protocols. Endophytic hydrocarbon-degrading bacteria may have a growth-promoting effect on the wild salt marsh halophyte plants and may be regarded as promising when field microbe-assisted phytoremediation approaches are envisaged.

Acknowledgments

This work was supported by the Centre for Environmental and Marine Studies, University of Aveiro (CESAM, project Pest C/MAR/LA0017/2011). Financial support to V. Oliveira was provided by the Portuguese Foundation for Science and Technology (FCT) in the form of a PhD grant (SFRH/BD/46977/2008).

Chapter 2

CHAPTER 2

Halophyte plant colonization as a driver of the composition of bacterial communities in salt marshes chronically exposed to oil hydrocarbons

Vanessa Oliveira, Newton C.M. Gomes, Daniel F.R. Cleary, Adelaide Almeida, Artur M. S. Silva, Mário M. Q. Simões, Helena Silva & Ângela Cunha

Accepted in FEMS Microbiology Ecology journal

Abstract: In this study, two molecular techniques [denaturing gradient gel electrophoresis (DGGE) and barcoded pyrosequencing] were used to evaluate the composition of bacterial communities in salt marsh microhabitats [bulk sediment and sediment surrounding the roots (rhizosphere) of *Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*] that have been differentially affected by oil hydrocarbon (OH) pollution. Both DGGE and pyrosequencing revealed that bacterial composition is structured by microhabitat. Rhizosphere sediment from both plant species revealed enrichment of OTUs (operational taxonomic units) closely related to *Acidimicrobiales*, *Myxococcales* and *Sphingomonadales*. The *in silico* metagenome analyses suggest that homologue genes related to OH degradation appeared to be more frequent in both plant rhizospheres than in bulk sediment.

In summary, this study suggests that halophyte plant colonization is an important driver of hydrocarbonoclastic bacterial community composition in estuarine environments, which can be exploited for *in situ* phytoremediation of OH in salt marsh environments.

Keywords: rhizosphere, pyrosequencing, microbial diversity, salt marshes

Introduction

Estuarine salt marshes are ecologically, commercially and economically important (Barbier *et al.*, 2011). Due to their high productivity and location, they provide valuable ecosystem services (Coulon *et al.*, 2012). However, being located between aquatic and terrestrial systems, they are vulnerable to perturbations from both environments (Bowen *et al.*, 2012). Although salt marsh conservation is generally believed to be important for

the maintenance of healthy coastal ecosystems, these ecosystems have been systematically destroyed due to urban and industrial growth (Reboreda & Caçador, 2007; Martins *et al.*, 2008). Anthropogenic inputs of hydrocarbons are one of the major threats to salt marsh vegetation (Carman *et al.*, 1996; Watts *et al.*, 2006; Martins *et al.*, 2008). Low molecular weight aromatic hydrocarbons are particularly deleterious and can affect plants during all growth stages (Gao & Zhu, 2004; Watts *et al.*, 2006; Watts *et al.*, 2008).

Microbial degradation is the primary route for the breakdown of hydrocarbons; degradation depends largely on composition and adaptive response to the presence of hydrocarbons (Leahy & Colwell, 1990). The use of plants and their associated microorganisms to promote bioremediation of degraded areas has been previously demonstrated in several studies (Siciliano & Germida, 1998; Daane *et al.*, 2001). Rhizosphere-associated microorganisms are currently regarded as pivotal in combating environmental contamination and there is growing interest in the development of new approaches for the mitigation of hydrocarbon contamination of coastal ecosystems (Daane *et al.*, 2001; Ribeiro e *et al.*, 2011). Although information exists regarding the microbial processes involved in hydrocarbon degradation (McGenity, 2014) many others questions, such as the organization of microbial community structure or even the mechanism involved in their adaptation to the presence of oil contamination, need additional data to implement appropriate bioremediation strategies. Molecular techniques provide an opportunity to understand microbial diversity and functionality in oil contaminated sites.

In a previous study, fluorescence in situ hybridization (FISH) was used to determine the relationship between different halophyte species and the relative abundance of prokaryote groups in salt marsh sediments and a metabolomics analysis revealed that volatile compounds released through the roots may underlie plant-bacteria associations in intertidal salt marshes (Oliveira *et al.*, 2012). Here we provide, for the first time, a thorough in-depth molecular characterization of bacterial communities in bulk sediment and rhizosphere microhabitats in a temperate estuary (Ria de Aveiro, Portugal) chronically exposed to oil hydrocarbon (OH) pollution. DGGE and barcoded pyrosequencing (16S rRNA gene amplicons) were used to assess how plant species- and OH pollution affect sediment bacterial composition in sampling sites exposed to

different levels of contamination. In addition to this, the 16S rRNA gene sequences retrieved in this study were used to generate *in silico* metagenomes and evaluate the distribution of potential bacterial traits in different microhabitats.

Material and methods

Sampling sites and sample processing

Samples were obtained from four sites of the Ria de Aveiro estuarine system (Aveiro, Portugal). The Ria de Aveiro is a shallow estuary, sometimes considered a coastal lagoon, on the northwest cost of Portugal (40.7° N, 8.7° W). It is formed by a complex network of channels and extensive intertidal zones (Dias *et al.*, 1999). Plant and sediment samples were taken during low tide, from four different sites (site A, 40˚35´52.64´´N, 8˚45´.0071´´W; site B, 40˚38´27.42´´N, 8˚44´15.42´´W; site C, 40˚37´32.18´´N, 8˚44´09.12´´W; site D, 40˚37´18.90´´N, 8˚39´46.28´´W (Fig. 2.1). In each site, four composite samples of each plant species (*Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*) were collected and stored separately in plastic bags for transportation. In the laboratory, bulk sediment and rhizosphere samples were separated. Roots were manually shaken to remove loosely bound sediment, which was discarded. The rhizosphere was extracted by suspending roots with tightly attached sediment particles in sterile deionized water. The mixture was kept in a rotary shaker for 30 min. and the resulting sediment slurry was decanted into a sterile Falcon tube and centrifuged (5 min., 7600 rpm, 4ºC). The supernatant was rejected (Phillips *et al.*, 2008). Bulk sediment and rhizosphere extracts

Fig. 2.1 Ria de Aveiro (Portugal) with the location of sampling stations (A, B, C or D).

were stored at -20ºC for subsequent molecular analysis. For salt marsh physicochemical characterization, three sediment subsamples were collected in each sampling site.

Sediments properties

For pH determination (Orion Model 290A), bulk sediment was suspended in water [1:5 (w/v)] (Faoun, 1984). Sub-samples were analyzed for organic matter content, as percentage of weight loss by ignition (8 h at 450 °C), moisture (expressed in percentage of water per quantity of fresh sediment weight) and sediment grain size (estimated from wet and dry sieving), following Quintino *et al.* (1989).

Hydrocarbon analysis

Bulk sediment was analyzed for aliphatic and aromatic hydrocarbons after a Soxhlet extraction followed by a gas chromatography mass spectrometry (GC-MS) analysis, conducted for three sediment sub-samples of each site that were previously homogenized and freeze-dried. For the standardization of the procedure, the internal calibration method was used in the quantification of 16 US EPA priority PAHs. Deuterated PAH surrogate standards were added to sediment samples in order to calculate the recovery efficiency during sample extraction, cleanup and analysis. For the aliphatic hydrocarbons fraction, two internal standards were used: undecane ($C_{11}H_{24}$) and tetracosane ($C_{24}H_{50}$).

Five grams of dried, homogenized sediment were extracted for 24 h in a Soxhlet apparatus with 150 mL of dichloromethane. Prior to extraction, the sediment was spiked with 25 µl (2 µg ml^{-1}) deuterated surrogate standards (naphthalene-d_8, acenapthlene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12}). Activated copper (Schubert, 1998) was added to the collection flask to avoid sulfur interferences in gas chromatography-mass spectrometry. The extracts were concentrated by rotary evaporation up to a volume of about 2-3 ml. The solvent was changed to 10 ml of hexane and the volume was further reduced to approximately 1-2 ml. Hydrocarbons in the concentrated hexane extract were separated using a 2:1 alumina/silica gel column with anhydrous sodium sulfate overlaying the alumina in order to remove water. The column was used to clean-up and fractionate the extract. Elution was performed using 15 ml of hexane to obtain the first fraction (aliphatic hydrocarbons), followed by an elution with 30 ml of

dicloromethane/hexane (1:1). These two eluents containing the aromatic hydrocarbons (PAH) were combined for analysis. The sample volume was reduced to 1 ml by rotary vacuum evaporator and further to 0.2 ml with a gentle pure nitrogen stream. A known quantity (2 mg ml^{-1}) of the internal standard, hexamethylbenzene, was added prior to GC-MS.

GC-MS analyses were conducted in an Agilent Network GC system, namely an Agilent 6890 gas chromatograph equipped with a mass selective detector (MSD 5973). The selective ion mode (SIM) was used for aromatic hydrocarbons and the scan mode was used for aliphatic hydrocarbons. The hydrocarbons were separated using a VF-5MS fused silica column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). GC/MS operating conditions were as follows: injector and transfer-line temperatures were maintained at 300 ºC. The oven temperature program was initially isothermal at 60 ºC for 1 min, increased to 200 ºC at a rate of 10 ºC/min (hold for 2 min), and then increased at a rate of 5 ºC/min (hold 8 min) and kept isothermal at 300 ºC. Helium was used as carrier gas, at a flow rate of 1.3 ml min^{-1}. Aliquots of 1 µl were manually injected in the splitless mode with a 7.50 min solvent delay. For aromatic hydrocarbons, mass spectra were acquired at the electron impact (EI) mode at 70 eV. The mass scanning ranged between m/z 20 and m/z 500.

The surrogate recoveries added to sediment samples were 78 ± 18 % for naphthalene-d_8, 91 ± 19 % for acenaphthene-d_{10}, 114 ± 25 % for phenanthrene-d_{10}, 109 ± 24 % for chrysene-d_{12} and 120 ± 20 % for perylene-d_{10}.

Total community DNA extraction

Total community DNA (TC-DNA) was extracted from bulk sediment and rhizosphere samples (0.5 g) with the MoBio Ultraclean™ soil DNA kit (Cambio) following manufacturer's instructions.

PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE)

A nested PCR approach was used to amplify the 16S rRNA gene sequences from bulk and rhizosphere sediment (Gomes *et al.*, 2008), which was more efficient for amplification of 16S rRNA gene fragments from sediment suitable for DGGE analyses.

Briefly, in the first PCR the universal bacterial primers U27 and 1492R (Weisburg *et al.*, 1991) were used. Reaction mixtures (25 µL) contained 12.5 µl DreamTaq™ PCR Master Mix (Fisher Scientific), 0.1 µM of each primer, 80 µg ml^{-1} bovine serum albumin (BSA) and 1 µl of template DNA. The amplification conditions were as follows: 5 min of denaturation at 94 ºC, 25 cycles of 45 s at 94 °C, 45 s at 56 °C, and 1.5 min at 72 °C; the PCR was finished by an extension step at 72 °C for 10 min. The amplicons obtained were used as template for a second PCR with the bacterial DGGE primers 984F-GC and 1378R (Heuer *et al.*, 1997). The PCR reaction mixtures (25 µl) consisted of 12.5 µl DreamTaq™ PCR Master Mix (Fisher Scientific), 0.1 µM of each primer, 1 % (v/v) dimethyl sulfoxide (DMSO) and 1 µl of template DNA. PCR amplification conditions: initial denaturation (94 ºC for 4 min); 30 cycles of denaturation (95 ºC for 1 min), annealing (53 ºC for 1 min), and extension (72 ºC for 1.5 min) and a final extension (72 ºC for 7 min). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel containing 6-10% acrylamide with a gradient of 40-58% of denaturants. The run was performed in Tris-acetate-EDTA buffer at 60 °C at a constant voltage of 80 V for 16 h using the DCode System (Universal Mutation Detection System, Bio-Rad). The DGGE gels were silver stained (Heuer *et al.*, 2001). The processing of the scanned DGGE gels was carried out using Bionumerics software 6.6 (Applied Maths).

Barcoded-pyrosequencing

A barcoded pyrosequencing approach was used for the analysis of bacterial communities in bulk and rhizosphere sediments. Composite samples (four subsamples) of bulk or rhizosphere sediments from each sampling site were used for pyrosequencing analysis. Fragments of the bacterial 16S ribosomal RNA (rRNA) gene were sequenced for each sample with primers V3 Forward (5′-ACTCCTACGGGAGGCAG-3′) and V4 Reverse (5′-TACNVRRGTHTCTAATYC-3′) (Wang & Qian, 2009), 1x Advantage 2 Polymerase Mix (Clontech), 1x Advantage 2 PCR Buffer, 0.2 µM of each PCR primer, 0.2 mM dNTPs (Bioron), 5% DMSO (Roche Diagnostics GmbH) and 2 µl of genomic DNA template in a total volume of 25 µl. The PCR conditions were as follows: 4 min denaturation at 94°C, followed by 25 cycles of 94°C for 30 s, 44°C for 45 s and 68°C for 60 s and a final extension at 68°C for 10 min. Negative controls were included for

all amplification reactions. Electrophoresis of duplicate PCR products was undertaken on a 1% (w/v) agarose gel and the 470 bp amplified fragments were purified using AMPure XP beads (Agencourt) or, if more than the expected fragment was amplified, gel purified using High Pure PCR Product Purification Kit (Roche Diagnostics GmbH), according to manufacturer's instructions. The amplicons were quantified by fluorometry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences) at Biocant (Cantanhede, Portugal). Sequences generated in this study can be downloaded from the NCBI Short Read Archive (Study accession: SRP035868). Analysis of the pyrosequencing data was performed using previously described methods (Pires *et al.*, 2012; Cleary *et al.*, 2013; Polónia *et al.*, 2014 - see supplementary methods for a detailed description).

Data analysis

Two square matrices were imported into R (R Core Team, 2013) using the read.table() function: 1) containing the presence and raw abundance of all OTUs per sample generated with Qiime and 2) containing band 'abundance' based on band intensity and position of the DGGE gel. In the OTU abundance matrix, sequences not classified as bacteria or classified as chloroplasts or mitochondria were removed prior to statistical analysis. Both matrices were log10 (x+1) transformed and a distance matrix was constructed using the Bray-Curtis index with the vegdist() function, in the vegan package (Oksanen *et al.*, 2011) in R. The Bray-Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Legendre & Gallagher, 2001; Cleary, 2003; Cleary *et al.*, 2013). Variation in OTU composition among microhabitats was assessed with Principal Coordinates Analysis (PCO) using the cmdscale() function in R with the Bray-Curtis distance matrix as input. We tested for significant variation in composition among microhabitats using the adonis() function in vegan. In the adonis analysis, the Bray-Curtis distance matrix of species composition was the response variable with biotope as independent variable. In DGGE Bray-Curtis distance matrix each biotope was the response variable with sampling sites as independent variable. The number of permutations was set at 999; all other arguments used the default values set

in the function. Weighted averages scores were computed for OTUs on the first two PCO axes using the wascores() function in the vegan package. Significant differences among microhabitats in the relative abundance of the most abundant bacterial phyla, classes and selected orders and the dominant OTU in each microhabitat were tested with an analysis of deviance using the glm() function in R. Because data were proportional, a glm with the family argument set to binomial was firstly applied. Because the ratio of residual deviance to residual d.f. in the models substantially exceeded 1, family was set to 'quasibinomial'. In the 'quasibinomial' family, the dispersion parameter is not fixed at 1 so that it can model over-dispersion. Using the glm model, we tested for significant variation among microhabitats using the anova() function in R with the F test, which is more appropriate when dispersion is estimated by moments, as is the case of quasibinomial fits.

Phylogenetic tree

Selected sequences from dominant OTUs (> 150) and their closest relatives retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). These were aligned and a bootstrap consensus tree was built with 500 replicates with MEGA 5 (http://www.megasoftware.net/). The bootstrap value represents the percentage of replicate trees in which the associated taxa clustered together. For tree inference, the nearest neighbor interchange (NNI) heuristic method and automatic initial tree selection were used. All positions containing gaps and missing data were eliminated.

In silico metagenome analysis

In the present study, PICRUSt (Langille *et al.*, 2013) was used to predict the metagenome of each sample. PICRUSt is a bioinformatics tool that uses marker genes, in this case 16S rRNA, to predict metagenome gene functional content. These predictions are pre-calculated for genes in databases including KEGG (Kyoto Encyclopedia of Genes and Genomes) and COG (Clusters of Orthologous Groups of proteins). In this study, the KEGG database and focused on KOs in the polyaromatic degradation pathway was used. R was used to generate bargraphs showing the relative abundance of total genes for each sample. Significant differences among biotopes in the relative abundance of total genes were tested with an analysis of deviance using the

glm() function in R. The glm model, was used to test for significant variation among biotopes using the anova() function in R with the F test, which is more appropriate when dispersion is estimated by moments, as is the case of quasibinomial fits.

Results and Discussion

Sediment properties and hydrocarbon contamination

The sediments of four sites were analyzed comparatively based on their physicochemical properties (Table 2.1) and on the quantification and composition of aliphatic and aromatic hydrocarbons (Table 2.2). Texture sediment characterization classified all sediments as mud, with the exception of sediment from site B (Table 2.1). In terms of total hydrocarbons, the concentration calculated as the sum of partial concentrations (aliphatic and 16 PAHs) ranged from 5.86 to 21.39 ng g^{-1} dry weight (dw), with the highest concentrations observed in site C and the lowest in site B. Sites A and D presented similar levels of overall hydrocarbon contamination (11.05 and 11.47 ng g^{-1} dw, respectively). Aliphatic hydrocarbons consisted mainly of C_{10}-C_{32} n-alkanes with total concentration ranging from 3.789 to 10.253 ng g^{-1} dw (Table 2). The total concentration of 16 PAHs in sediment ranged from 1.285 (A) to 11.134 (C) ng g^{-1} dw (Table 2.3).

Table 2.1. Sediment properties in four salt marshes sites of a estuarine system Ria de Aveiro (main value ± STD, n = 3).

	Sampling sites			
	A	B	C	D
pH	7.12 ± 0.10	8.34 ± 0.20	7.49 ± 0.26	7.24 ± 0.24
Organic matter (%)	4.54 ± 1.13	2.67 ± 1.15	5.81 ± 2.08	7.20 ± 2.97
Moisture (%)	22.47 ± 5.03	17.74 ± 3.18	36.61 ± 8.18	40.10 ± 10.33
% Fines	58.06 ± 0.06	20.96 ± 0.06	81.99 ± 0.13	62.67 ± 0.03
Sediment Texture	Mud	Very fine sand	Mud	Mud

The localization of the sampling points may explain to some extent the differences in concentration of hydrocarbons obtained. Sites A and C are located in the Mira channel, one of the four main channels of the estuarine system, whereas sites B and D are located in secondary channels. Another important feature of site C, the most

contaminated site, is that it is located close to the Port of Aveiro and exposed to greater anthropogenic activity (recreational, navigation, urban runoff and shipping activity). Because hydrocarbons may have multiple origins, some aliphatic diagnostic indices were used to identify biogenic (terrestrial and marine) and/or anthropogenic sources. Pristane (C_{19}) and phytane (C_{20}) are common isoprenoids in coastal marine sediments and good indicators of petroleum contamination (Readman *et al.*, 2002). The pristane to phytane ratios (Pr/Ph) of ≤ 1 reflect petroleum contamination and higher ratios indicate biogenic source. In this study, the Pr/Ph ratios (Table 2.2) indicate that hydrocarbons in sediments are most likely associated to petroleum contamination in sites A, C and D and are predominantly of biogenic origin at site B. Other aliphatic indicators, such as n-C_{17}/Pr and n-C_{18}/Ph ratios (Table 2.2), are useful as indicators of early microbial degradation (Díez *et al.*, 2007). Generally, lower n-C_{17}/Pr ratios (<1) reflect the relative contribution of allochthonous and autochthonous hydrocarbons to the sediment (Mille *et al.*, 2007). The calculated n-C_{18}/Ph ratios in sediments from sites A and C were low (0.78–1.14), which indicates that microbial biodegradation of n-alkanes is an important process at these sites (Díez *et al.*, 2007).

Table 2.2. Concentration of aliphatic hydrocarbons (ng g^{-1} dry weight) and values of selected source diagnostic indices in four salt marsh sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3).

	Sampling sites			
Aliphatics (n-C_{10}-C_{32}) (ng g^{-1} dry weight)	A	B	C	D
nC_{17}	0.310 ± 0.130	0.160 ± 0.068	0.497 ± 0.332	0.491 ± 0.299
nC_{18}	0.528 ± 0.068	0.370 ± 0.018	1.084 ± 0.188	1.170 ± 0.257
Pr (nC_{19})	0.382 ± 0.103	0.326 ± 0.014	0.509 ± 0.161	0.474 ± 0.268
Ph (nC_{20})	0.681 ± 0.305	0.235 ± 0.230	0.947 ± 0.226	0.813 ± 0.341
Σ Aliphatics (n-C_{10}-C_{32})	9.764 ± 0.171	3.789 ± 0.058	10.253 ± 0.140	9.527 ± 0.142
Pr/Ph	0.562	1.390	0.537	0.582
nC_{17}/Pr	0.812	0.490	0.977	1.037
nC_{18}/Ph	0.776	1.577	1.145	1.438
Σ Total HC	11.050 ± 0.111	5.859 ± 0.006	21.386 ± 0.002	11.471 ± 0.068

Pr, Pristine; Ph, Phytane; Total HC, Total hydrocarbon = sum of total Aliphatics (n-C_{10}-C_{32}) + Σ 16 PAH

The composition of the sediment PAH pool showed some differences between sites. There was a marked predominance of 5-6 ring PAHs at sites B, C and D but 2-3 ring PAHs were most abundant in sediment from site A (Table 2.3). This pattern may be related with the prevailing conditions at different estuarine sites since high-molecular-weight PAHs tend to predominate in sediments from marine and river environments (Yan *et al.*, 2009; Guo *et al.*, 2011; Commendatore *et al.*, 2012; Gonul & Kucuksezgin, 2012). PAH molecular indices based on the ratios of selected PAH concentrations may help to infer the pyrogenic (originated from the combustion of fuels) or petrogenic (originated from petroleum) origin of the PAH pool (Budzinski *et al.*, 1997).

Table 2.3 Concentration of PAH (ng g^{-1} dry weight) in four salt marsh sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3).

		Sampling sites			
	PAH (ng g^{-1} dry weight)	A	B	C	D
2- to 3-ring	Naph	0.207 ± 0.049	0.204 ± 0.091	0.279 ± 0.092	0.205 ± 0.059
	Aceph	0.049 ± 0.002	0.053 ± 0.004	0.121 ± 0.021	0.054 ± 0.004
	Ace	0.048 ± 0.004	0.046 ± 0.001	0.051 ± 0.002	0.045 ± 0.001
	Flu	0.061 ± 0.004	0.052 ± 0.003	0.111 ± 0.008	0.051 ± 0.004
	Phe	0.090 ± 0.012	0.120 ± 0.031	0.710 ± 0.193	0.091 ± 0.013
	Ant	0.061 ± 0.001	0.070 ± 0.011	0.370 ± 0.243	0.059 ± 0.002
4 ring	Fluor	0.081 ± 0.021	0.186 ± 0.128	1.502 ± 0.185	0.116 ± 0.039
	Pyr	0.071 ± 0.019	0.163 ± 0.105	1.155 ± 0.120	0.102 ± 0.031
	BaA	0.069 ± 0.015	0.128 ± 0.063	0.832 ± 0.217	0.118 ± 0.052
	Chr	0.056 ± 0.009	0.099 ± 0.060	0.474 ± 0.088	0.089 ± 0.024
5- to 6-ring	BbF	0.096 ± 0.039	0.188 ± 0.097	1.330 ± 0.257	0.228 ± 0.097
	BkF	0.066 ± 0.007	0.111 ± 0.046	0.307 ± 0.050	0.089 ± 0.020
	BaP	0.098 ± 0.022	0.268 ± 0.170	2.032 ± 0.551	0.288 ± 0.176
	InP	0.097 ± 0.029	0.167 ± 0.066	0.996 ± 0.184	0.183 ± 0.073
	DahA	0.064 ± 0.005	0.077 ± 0.008	0.161 ± 0.041	0.074 ± 0.009
	BghiP	0.073 ± 0.007	0.139 ± 0.051	0.702 ± 0.114	0.150 ± 0.062
	Σ 16 PAH	1.285 ± 0.014	2.071 ± 0.050	11.134 ± 0.136	1.944 ± 0.046

Naph, Naphthalene; Aceph, Acenaphthylene; Ace, Acenaphthene; Flu, Fluorene; Phe, Phenanthrene; Ant, Anthracene; Fluor, Fluoranthene; Pyr, Pyrene; BaA, Benzo(a)anthracene; Chr, Chrysene; BbF, Benzo(b)fluoranthene; BkF, Benzo(k)fluoranthene; BaP, Benzo(a)pyrene; InP, Indeno(1,2,3-cd) pyrene; DahA, Dibenz(a,h) anthracene; BghiP, Benzo(g,h,i) perylene

According to the diagnostic criteria (Table 2.4), PAHs found in the sediments of Ria de Aveiro are associated to fossil fuel combustion (pyrogenic sources). The ratio of low-molecular-weight (LMW) to high-molecular-weight (HMW) ranged from 0.173 to 0.669 (Table 2.4). Taken together with the Phe/Ant, Fluor/Pyr, Fluor/(Fluor+Pyr) and InP/(InP+BghiP) ratios (Luo *et al.*, 2005), the results confirm that the PAH contamination of salt marsh sediments of Ria de Aveiro is typically related to chronic fossil fuel exposure. In a recent report, the effect and bioavailability of PAHs in three different marine sediments (muddy, sand and organic) were tested (Lindgren *et al.*, 2014). Their results show that muddy sediment, although containing the highest total PAH concentrations after 60 days, had the lowest bioavailable concentration. On the other hand, sandy sediment contained the lowest total PAH concentrations but the highest bioavailability. In this study, the sandy sediment (site B) has the lowest total hydrocarbon concentration, related to the higher bioavailability characteristic of this type of sediment.

Table 2.4 Origin-indicative PAH ratios and ratio values calculated for this study.

Ratio	LMW/HMW	Phe/Ant	Fluor/Pyr	Fluor/(Fluor+Pyr)	InP/(InP+BghiP)
Pyrolytic source					
	<1	<10	>1	>0.5	>0.5
Petrogenic source					
	>1	>15	<1	<0.5	<0.2
References	Soclo *et al.*, 2000; Tam *et al.*, 2001; Magi *et al.*, 2002	Budzinski *et al.*, 1997; Baumard *et al.*, 1998; Baumard *et al.*, 1999	Budzinski *et al.*, 1997; Baumard *et al.*, 1998; Baumard *et al.*, 1999	Gogou *et al.*, 1998	Yunker *et al.*, 2002
Sampling sites					
A	0.669	1.488	1.135	0.532	0.572
B	0.358	1.716	1.140	0.533	0.546
C	0.173	1.917	1.300	0.565	0.587
D	0.351	1.540	1.137	0.532	0.549

LMW, Low-molecular weight; HMW, High-molecular-weight; Phe, Phenanthrene; Ant, Anthracene; Fluor, Fluoranthene; Pyr, Pyrene; InP, Indeno(1,2,3-cd) pyrene; BghiP, Benzo(g,h,i) perylene

Site-related variation of the structure of microbial communities

DGGE fingerprinting analysis revealed a significant association between composition of bacterial communities and sampling site, in the microhabitats corresponding to the rhizosphere of *H. portulacoides* (Adonis $F_{3,15} = 5.07$, $P < 0.001$, $R^2 = 0.559$), the rhizosphere of *S. perennis* subsp. *perennis* (Adonis $F_{3,15} = 4.57$, $P < 0.001$, $R^2 = 0.533$) and bulk sediment (Adonis $F_{3,15} = 4.18$, $P < 0.001$, $R^2 = 0.511$). These differences can also be observed in the PCO ordinations present in Fig. 2.2. The first two PCO axes explained approximately 50 % of the variation, with samples of each sampling site clustering together for each microhabitat.

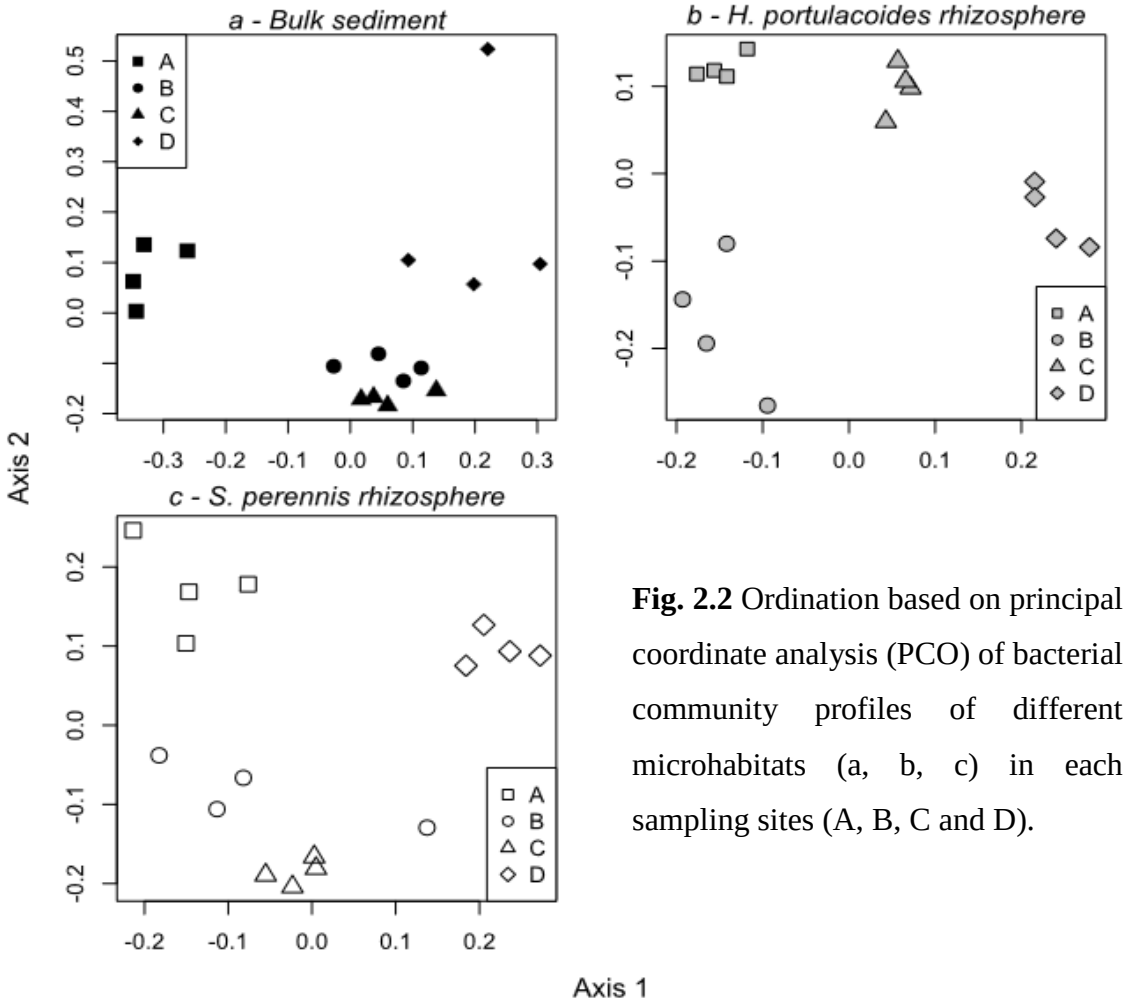

Fig. 2.2 Ordination based on principal coordinate analysis (PCO) of bacterial community profiles of different microhabitats (a, b, c) in each sampling sites (A, B, C and D).

In salt marsh sediments, the composition of the community in terms of the dominant microbial taxa is structured by local factors (Bowen *et al.*, 2009) related with geographical localization, environmental conditions and pollutants (Córdova-Kreylos *et al.*, 2006). Studies conducted in the Ria de Aveiro have suggest that environmental factors structure bacterial communities (Cleary *et al.*, 2012; Oliveira *et al.*, 2012). In this study, localization of the vegetation banks in the complex estuarine system and the differential exposure to environmental contamination, together with different physicochemical characteristics and hydrodynamics of each sampling site, might have influenced bacterial composition. The geographical location of site C, close to Port of Aveiro, may explain the higher organic matter content and hydrocarbon concentration in the sediment, as compared with the other sites. Site B is located in a secondary channel, more sheltered from direct sources of contamination or intense anthropogenic activities. In this case, other physicochemical characteristics, and a different sediment texture (Table 2.1), were probably major determinants of bacterial composition.

In this study, hydrocarbon concentrations were lower than reported in other salt marshes (Hwang *et al.*, 2006; Watts *et al.*, 2006; Ribeiro *et al.*, 2011). Previous studies have, however, shown that relatively low levels of hydrocarbons in the sediment had an effect on the structural diversity of bacterial communities (Castle *et al.*, 2006; Labbé *et al.*, 2007).

Structure of bacterial communities in salt marsh sediments

The structure of sediment bacterial communities associated to *H. portulacoides* and *S. perennis* subsp. *perennis* roots and bulk salt marsh sediments were determined by 16S rRNA gene amplicons. *Proteobacteria* represented approximately 60 % of the total sequences in all samples but *Actinobacteria* were also well represented in rhizosphere samples (Fig. 2.3). *Bacteroidetes* were the third most dominant group, accounting, on average, for 8.3 % of the sequences. These three phyla were the most abundant groups detected in a study conducted in a coastal salt marsh during and after the influx of petroleum hydrocarbons following the Deepwater Horizon oil spill (Beazley *et al.*, 2012). The phylum *Proteobacteria* is considered a dominant group in estuarine sediment samples (Bowen *et al.*, 2012; Gomes *et al.*, 2013; Gomes *et al.*, 2014). Within *Proteobacteria*, *Alphaproteobacteria* were the most dominant class in rhizosphere

samples ($F_{2,9}$ = 10.745 p = 0.004) whereas *Gammaproteobacteria* was the most abundant class in bulk sediment (Fig. 2.3).

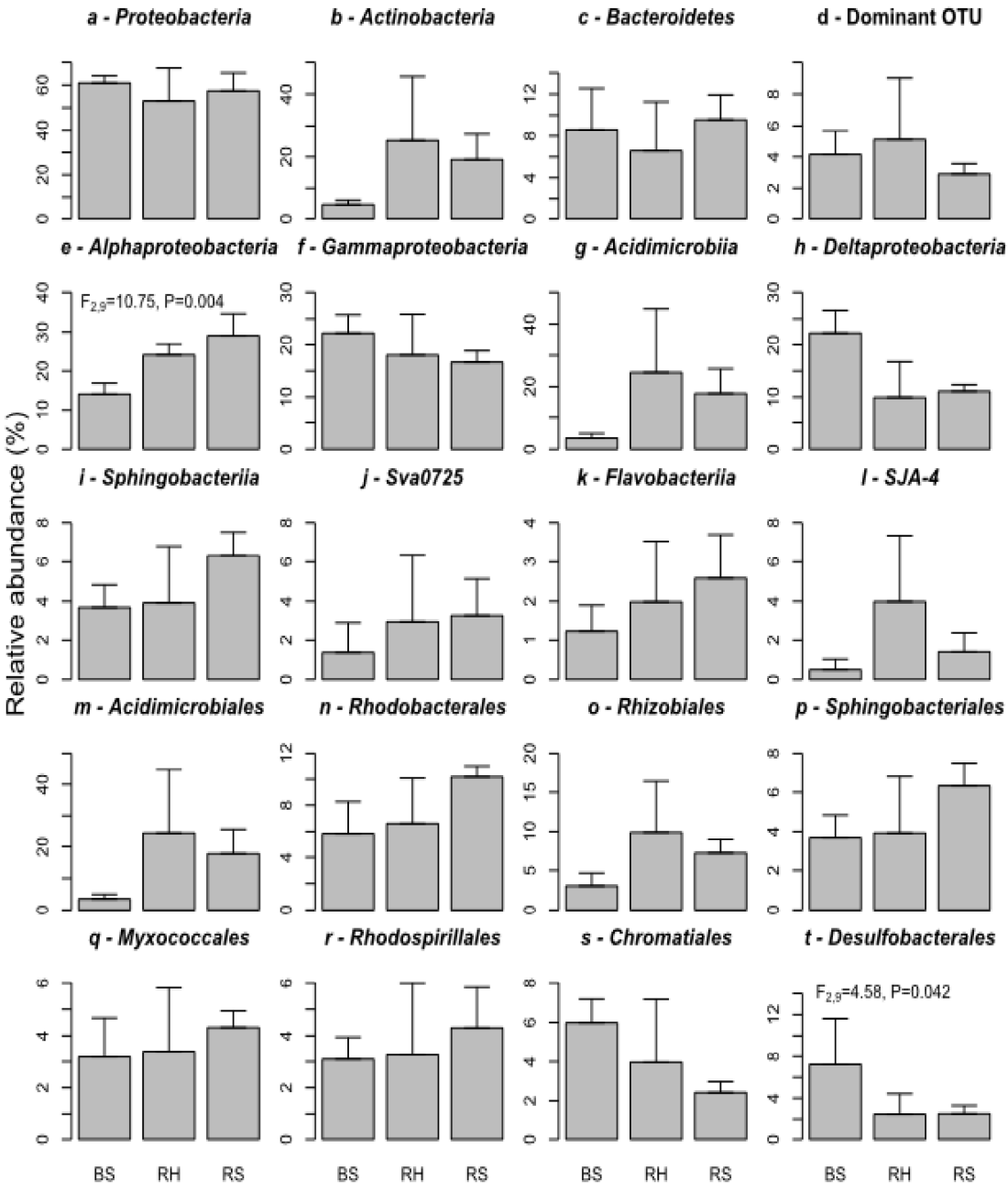

Fig. 2.3 Relative abundance of the most represented bacterial taxa: three most abundant phyla (a, b, c), eight most abundant classes (e, f, g, h, i, j, k, l), the eight most abundant orders (m, n, o, p, q, r, s, t) and the most dominant OTU (d) in bulk sediments (BS), *H. portulacoides* rhizosphere (RH) and *S. perennis* rhizosphere (RS).

The distribution of *Alphaproteobacteria* in marine and freshwater environments is well documented and hydrocarbonoclastic *Alphaproteobacteria* have been detected in marine environments (Kim & Kwon, 2010; Newton *et al.*, 2011). On the other hand, *Gammaproteobacteria* have been reported as abundant in coastal sediment, namely Atlantic port sediments (Gomes *et al.*, 2013), Eastern Mediterranean Sea sediments (Polymenakou *et al.*, 2005), Northeastern Pacific (Kouridaki *et al.*, 2010) and South China Sea sediments (Zhu *et al.*, 2013). In bulk sediment, *Deltaproteobacteria* was the second most dominant class (Fig. 2.3), immediately following *Gammaproteobacteria*. The high relative abundance of the *Desulfobacterales* order in bulk sediment ($F_{2,9}$ = 4.585 p = 0.042) may be a result of more anaerobic conditions in bulk sediment which in turn could select for specific guilds such as sulfate reducing bacteria (SRB). These results are consistent with other studies conducted in the same estuarine system that revealed that the SRB order *Desulfobacterales* was the most abundant group in unvegetated sediment (Gomes *et al.*, 2010a; Cleary *et al.*, 2012). In these sediments, generally anoxic just below the surface and suboxic to oxic at the surface (Cunha *et al.*, 2005; Santos *et al.*, 2007), anaerobic metabolism such as fermentation and anaerobic respiration, may represent the major pathways of organic matter oxidation. The high relative proportion of SRB in salt marsh sediments can be explained by the availability of fermentation-derived substrates directly utilized by SRB (Hines *et al.*, 1999). Moreover, sulfate-reducing strains capable of growing and degrading hydrocarbons have been isolated from hydrocarbon-polluted marine sediment (Cravo-Laureau *et al.*, 2004), and some strains were capable of oxidizing alkenes (Aeckersberg *et al.*, 1991; Cravo-Laureau *et al.*, 2004). SRB play critical roles in a variety of processes in coastal marine sediments such as, organic matter turnover, biodegradation of pollutants, sequestration of metals, and sulfur and carbon cycles (Zhang *et al.*, 2008). Also, recent studies showed that the relative abundance of the order *Desulfobacterales* increases in marine sediments contaminated with OH (Suárez *et al.*, 2011; Acosta-González *et al.*, 2013). A phylogenetic tree of selected dominant OTUs (> 150 sequence reads) and their closest relatives was constructed (Fig. 2.4).

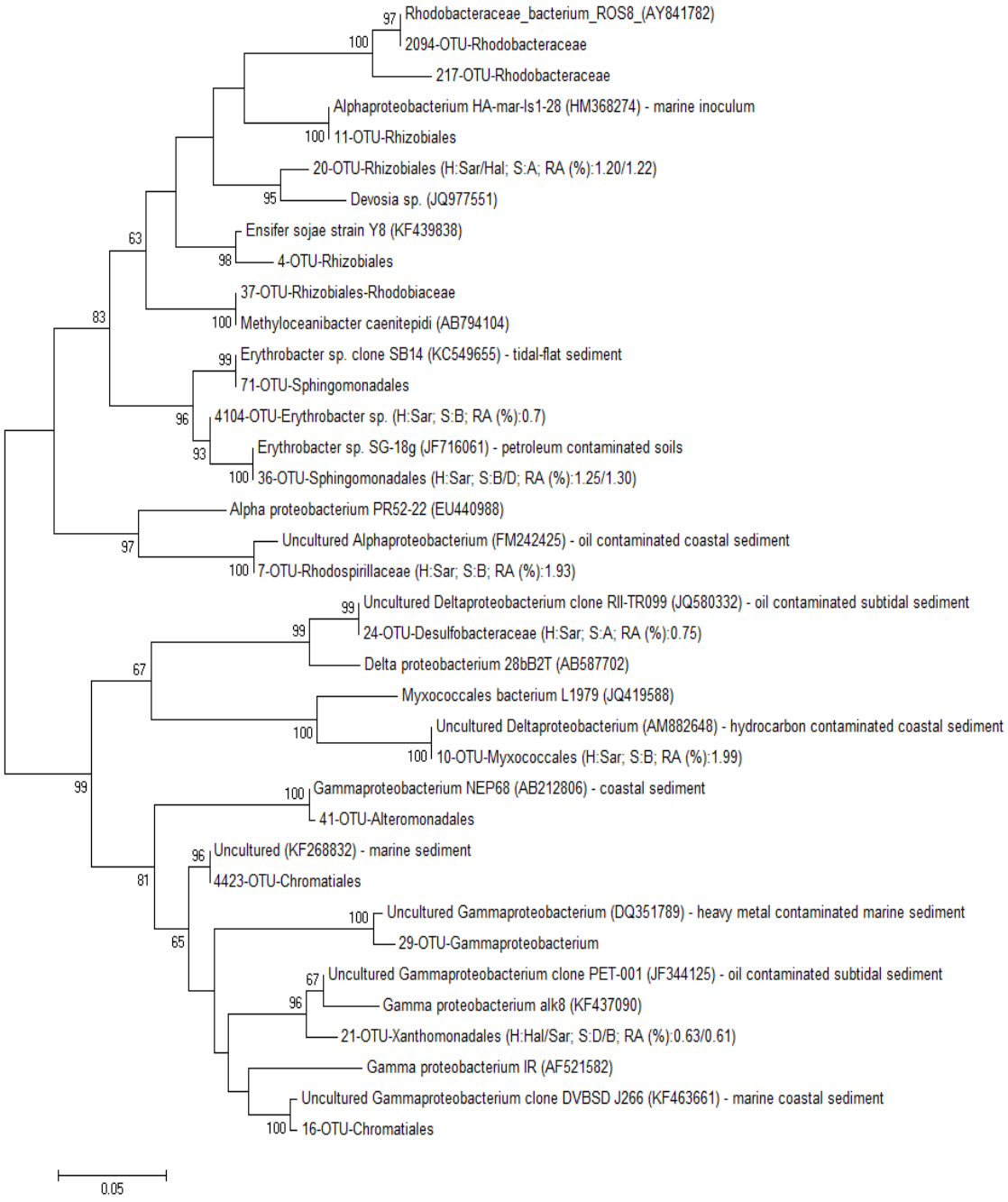

Fig. 2.4 Phylogenetic tree showing the *Proteobacteria* (A) and *Actinobacteria-Acidobacteria-Bacteriodetes* (B) clusters from bulk sediment and salt marsh rhizospheres of *H. portulacoides* and *S. perennis*. Close relatives of the selected sequences were obtained by using the BLAST search. H: Microhabitat (rhizosphere samples from *Halimione portulacoides* (Hal) and *Sarcocornia perennis subsp. perennis* (Sar)); S: Sampling site (A, B, C or D); RA: Relative abundance of the respective OTU.

The phylogenetic analysis revealed bacterial phylotypes closely related with phyla *Proteobacteria*, *Actinobacteria* and *Acidobacteria.* Only one abundant OTU (18) was associated with bulk sediment. This OTU was classified as belonging to the *Acidobacteria* class and closely related to an uncultured bacterium (AF523900) isolated from forest wetland impacted by coal (Brofft *et al.*, 2002). Most bacterial populations in both rhizospheres showed phylogenetic affiliation with ecotypes from a wide diversity of marine environments. Moreover, the majority of these ecotypes are related to hydrocarbon impacted sediments (Fig. 2.4).

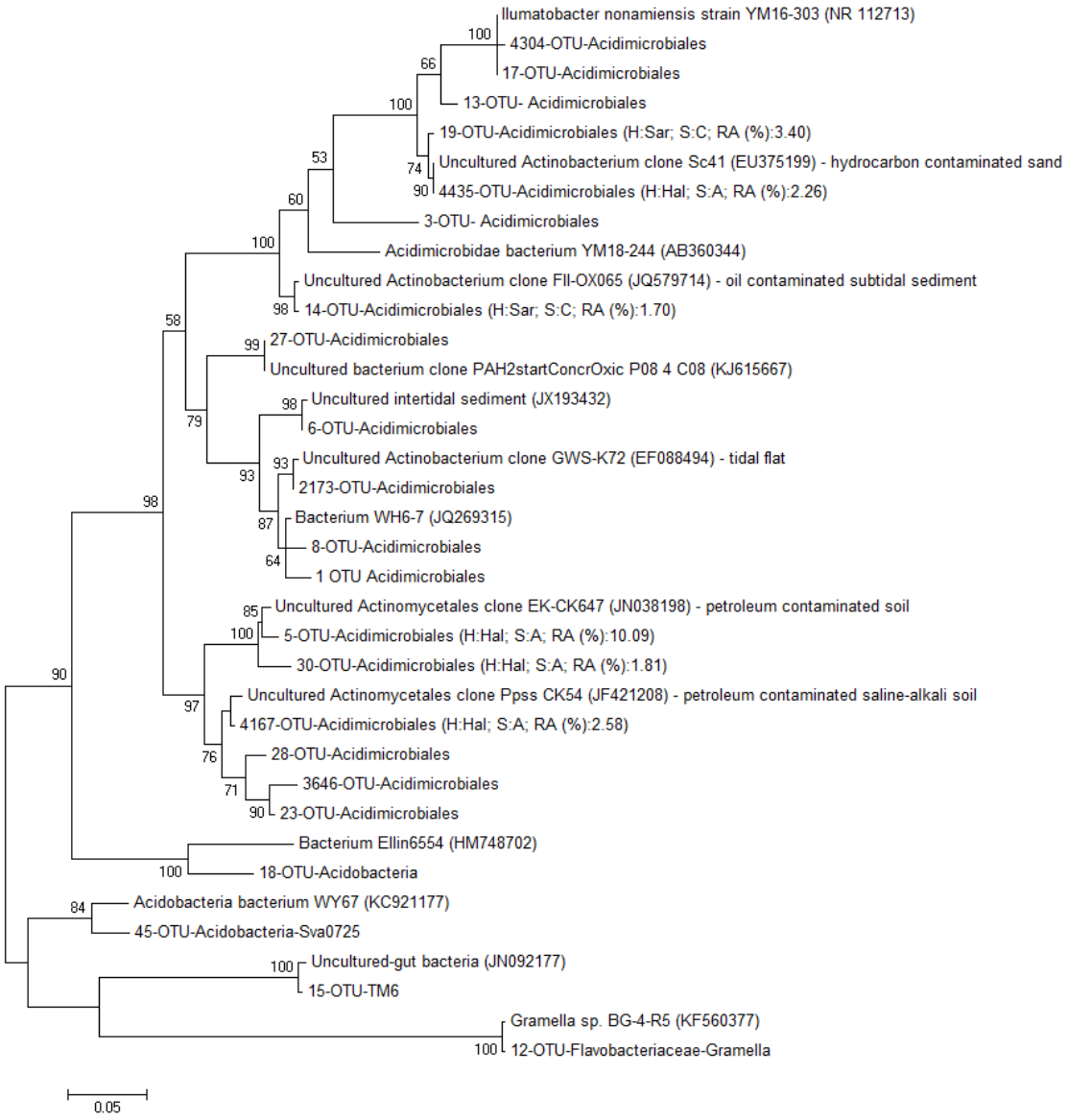

Fig. 2.4 (continued).

A study performed after the *Prestige* oil spill revealed that members of the classes *Alphaproteobacteria* and *Actinobacteria* were the prevailing groups of bacteria in shoreline environments (Alonso-Gutiérrez *et al.*, 2009). Also, a predominance of *Gamma-* and *Deltaproteobacteria* in anaerobic bacteria communities of coastal sediments was reported after the *Prestige* oil spill (Acosta-González *et al.*, 2013). The phylogenetic analysis showed that some OTUs associated with *S. perennis* rhizosphere were closely related to gut bacteria (OTU 15) and uncultured *Acidobacteria* from coastal soils (OTU 45; Fig. 2.4A).

Plant-related variation in the structure of bacterial communities

Overall analysis of the barcoded pyrosequencing data, comparing all rhizosphere samples against bulk sediment samples, showed significant differences between bacterial communities from bulk sediment and rhizospheres (Adonis $F_{2,11} = 2.09$, $P < 0.001$, $R^2 = 0.317$). Shannon indices indicate that bacterial communities of *S. perennis* subsp. *perennis* rhizosphere were more diverse than *H. portulacoides* rhizosphere and bulk sediment (Table 2.S1). The PCO ordination of bacterial operational taxonomic units (OTUs; Fig. 2.5) indicates that the two rhizospheres shared the most abundant OTUs (large grey circles; > 150 sequences). The rhizosphere effect can explain the higher number of OTUs in these samples, when compared to bulk sediment, and underlies an apparent convergent adaptation of rhizosphere communities. Plant-bacteria interactions, exerted through the release of exudates that create a unique physicochemical environment surrounding roots, explained the different shifts in bacterial community composition between rhizospheres and bulk sediment. The halophyte exudates provide oxygen and high-quality sources of carbon and energy for bacterial growth (Bagwell *et al.*, 1998). Moreover, roots provide physical support and distinct chemical conditions (Singh *et al.*, 2004; Mucha *et al.*, 2010) and the presence of plants differentially promotes the development of some bacterial groups (Berg & Smalla, 2009; Mucha *et al.*, 2011; Gomes *et al.*, 2014).

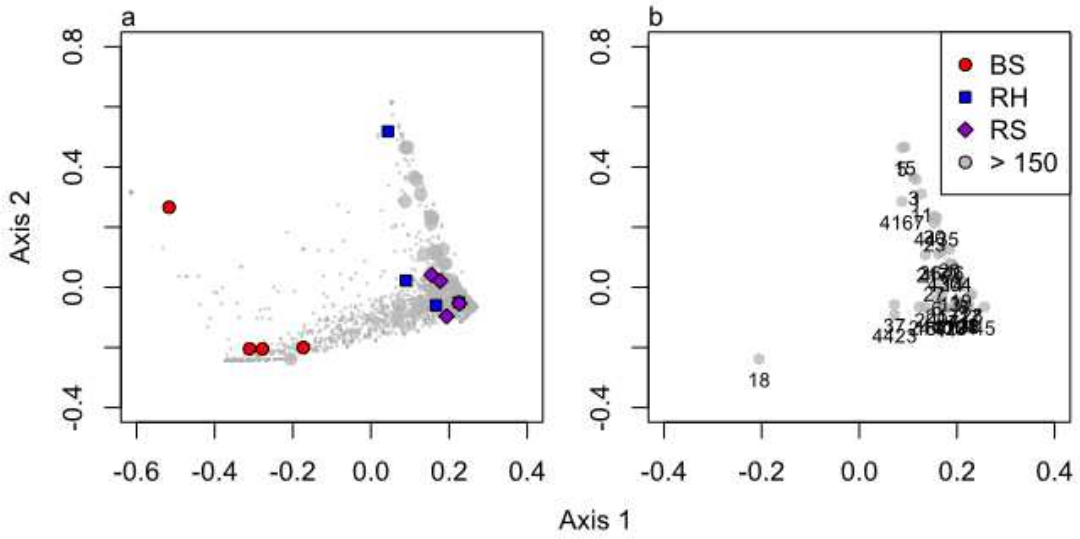

Fig. 2.5 (a) Ordination based on principal coordinate analysis (PCO) of pyrosequencing data. Samples are represented by symbols (BS – Bulk sediment; RH – *H. portulacoides* rhizosphere and RS – *S. perennis* rhizosphere). Operational taxonomic unit (OTU) are represented by circles and OTUs with > 150 sequences are represented by large circles. (b) OTU number of dominant 16S rRNA sequence reads (> 150 sequences).

As previously mentioned, *Actinobacteria* and *Gammaproteobacteria* were more abundant in rhizospheres than bulk sediment and the oxic conditions around the roots may explain a lower relative abundance of *Desulfobacterales*, and consequently *Deltaproteobacteria*, in relation to bulk sediment (Fig. 2.3). The orders *Acidimicrobiales*, *Rhodobacterales*, *Rhizobiales*, *Sphingobacteriales* and *Rhodospirillales* were also more abundant in rhizospheres than in bulk sediment (Fig. 2.3). The rhizosphere of *H. portulacoides* showed enrichment of *Rhizobiales* and *Acidimicrobiales. Rhizobiales* members are involved in atmospheric nitrogen fixation by plants and some members of this group have also been reported as degraders of aromatic hydrocarbon compounds (Baek *et al.*, 2003). *Acidimicrobiales* members have been considered to be responsive to changes in soil pH (Lauber *et al.*, 2009). Members of the *Actinobacteria* phylum capable of degrading hydrocarbons were isolated from Artic native plant species (Ferrera-Rodríguez *et al.*, 2013). The rhizosphere of *S. perennis* subsp. *perennis* showed enrichment of *Rhodobacterales*, *Sphingobacteriales* and *Rhodospirillales*. *Rhodobacterales* have been associated with the degradation of

aliphatic and low-molecular weight aromatic hydrocarbon (Harwati *et al.*, 2007) and the association of *Sphingobacteriales* and *Rhodospirillales* members with PAH and petroleum degradation in soil has also been previously reported (Gomes *et al.*, 2010b; Mao *et al.*, 2012).

Phylogenetic analysis of the dominant selected OTUs (> 150 sequence reads) showed that thirteen of these OTUs, predominant in rhizosphere samples, are closely related to ecotypes of OH impacted sediments (Fig. 2.4). Six OTUs (5, 14, 19, 30, 4167 and 4435) were related to organisms assigned to the *Acidimicrobiales* order, which were previously reported from oil-polluted subtidal sediments (Acosta-González *et al.*, 2013) as well as from fuel pollution-affected shoreline environments (Alonso-Gutiérrez *et al.*, 2009). OTUs 19 and 14 were enriched in the rhizosphere of *S. perennis* subsp. *perennis* in the most contaminated sampling site (C), and OTUs 5, 30, 4167 and 4435 in the rhizosphere of *H. portulacoides* in sampling site A where the aliphatic hydrocarbon concentration is higher (Fig. 2.4B; Table 2.2). The other seven OTUs (7, 10, 20, 21, 24, 36 and 4104) were predominantly enriched in the rhizosphere of *S. perennis* subsp. *perennis* in site B, the second most polluted site (Fig. 2.4A; Table 2.3). These OTUs are phylogenetically closely related to proteobacterial orders that are known OH degraders. In this study, OTUs 10 and 24 were assigned to *Myxococcales* and *Desulfobacteraceae*, respectively. Both of these OTUs are affiliated to sulfate reducing groups that may play an important role in hydrocarbon degradation in contaminated coastal sediments (Paisse *et al.*, 2008; Acosta-González *et al.*, 2013). Members of *Xanthomonadales* (OTU 21), *Rhodospirillaceae* (OTU 7) and *Rhizobiales* (OTU 20) that were detected in this study were associated with organisms related to either alkane degradation or oil-polluted sites (Viñas *et al.* 2005; Alonso-Gutiérrez *et al.* 2009; Patel *et al.*, 2012). According to the phylogenetic analysis, the OTU 2094 was clustered into family *Rhodobacteraceae.* Members of this family have highly diverse metabolism and includes hydrocarbonoclastic species from freshwater and marine environments (Chang *et al.*, 2000; Brakstad & Lødeng, 2005). Two dominant OTUs (38 and 4104) showed close phylogenetic relationship to the genus *Erythrobacter*. Several marine oil-hydrocarbon degrading stains related to *Erythrobacter sp.* were found to contain cytochrome P450 CYP153 and *alkB* genes (Wang *et al.*, 2010). The *alk* genes code for an important enzyme involved in n-alkanes environmental degradation, and CYP153A genes were

detected in petroleum-contaminated soil, groundwater and coastal seawater (Kubota *et al*., 2005). Our phylogenetic analysis also showed a dominance of OTUs closely related to *Actinobacteria* in *H. portulacoides* rhizosphere, members with high G+C content, are gram-positive and are physiologically diverse. In this study, the OTUs sequences for *Acidimicrobiales* order were mostly phylogenetic associated with uncultured *Actinobacteria* members. These observations mirrored results from uncultured (Militon *et al.*, 2010) and culture-based (Pucci *et al.*, 2000) approaches suggesting that *Actinobacteria* (*Acidimicrobiales and Actinomycetales*) might play a role in the bioremediation of alkane-contaminated sediments. Autochthonous strains with the capacity to degrade alkanes were isolated from crude oil polluted sites (Pucci *et al.*, 2000). Moreover, it was observed that during bioremediation processes the active actinobacterial phylotypes increase (Militon *et al.*, 2010). The abundance of sequences phylogenetically related to hydrocarbon degrading organisms in the rhizosphere samples analyzed in this study provides evidence that halophyte plant colonization is an important driver of the structure of hydrocarbonoclastic bacterial communities in salt marshes and demonstrates the potential of plant-bacteria interactions in processes of microbe-assisted phytoremediation of hydrocarbons in coastal ecosystems.

In silico metagenome analysis

PICRUSt algorithm was used to predict metagenome functional content based on the Kyoto encyclopedia of genes and genomes (KEGG) classification to identify potential bacterial traits in different microhabitats. Figure 2.6 shows the relative gene count of selected KOs that might be involved in PAH degradation pathways (www.genome.jp/kegg-bin/show_pathway?ko00624). Significant differences from three selected KOs were observed. KOs K00449, K00517 and K00599, have a higher relative gene frequency in rhizosphere samples, but not KO K00492, a methyltransferase involved in the final pathway of phenanthrene and pyrene degradation. K00492, oxidoreductase, is important in the initial steps of the phenanthrene degradation pathway. The protocatechuate 3,4-dioxygenase (K00449) produced by *pcaH* is important in the final steps of the metabolism of several PAHs (fluorene, anthracene and phenanthrene). K00517 is an oxidoreductase that acts in the initial step of anthracene degradation to 9,10-anthraquinone. These selected KOs are not exclusive of

PAH degradation pathways but are involved in other metabolic pathways especially related to the degradation of aromatic compounds (www.genome.jp/kegg/).

Despite the fact that the data obtained here were derived from an *in silico* metagenome analysis, the functional genetic information obtained still represents an important resource for the initial evaluation of the distribution of potential bacterial traits in different microhabitats.

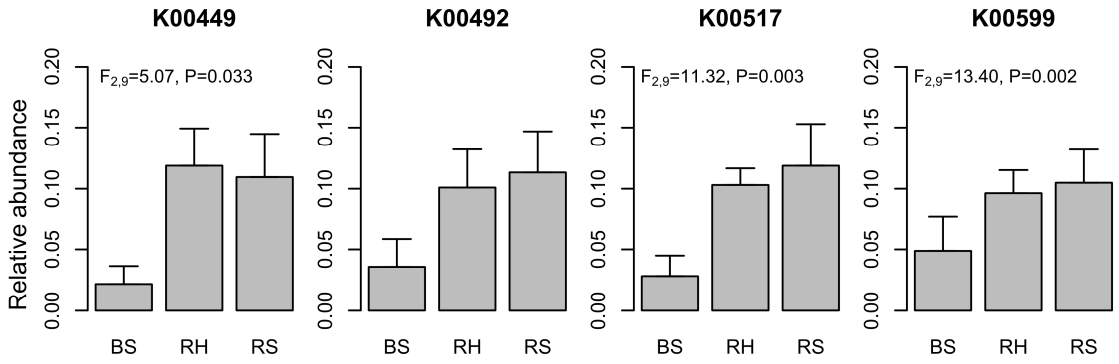

Fig 2.6 Relative gene count of selected KOs could possible involved in PAH degradation. Samples are represented by symbols (BS – Bulk sediment; RH – *H. portulacoides* rhizosphere and RS – *S. perennis* subsp. *perennis* rhizosphere). KOs enzyme terminology: K00449 - *pcaH*; K0042 - 1.14.13.- (Oxidoreductases); K00517 - E1.14.-.- (Oxidoreductases); K00599 - E2.1.1.- (Transferases).

Conclusion

Microbial communities in salt marsh sediments appear to respond to interplaying factors related to the physico-chemical characteristics of the environment, sediment properties, plant colonization and pollution. The results of this study demonstrate that the structure of bacterial communities is probably affected by site-related factors, such as sediment properties and hydrocarbon contamination, and by plant-related factors.

The barcoded pyrosequencing approach used in this study provided a comprehensive overview of the rhizosphere bacterial communities associated with two important halophyte plants from temperate estuaries. The results indicate that root systems of *H. portulacoides* and *S. perennis* subsp. *perennis* appear to be able to exert a strong influence on bacterial composition. The orders *Acidimicrobiales* and *Rhizobiales*

were both associated with the rhizospheres of both halophytes whereas the order *Desulfobacterales* was associated with bulk sediment. In addition to this, the *in silico* metagenome analysis revealed a possible higher number of genes involved in the process of PAH degradation in the rhizosphere of halophytic plants. This indicates that halophyte plant colonization could be an important driver of hydrocarbonovlastic bacteria community structure in salt marsh sediments, which can be exploited for *in situ* phytoremediation of OH in salt marsh environments.

Acknowledgements

This work had financial support from CESAM research unit (Pest C/MAR/LA0017/2011), QOPNA research unit (PEst-C/QUI/UI0062/2013; FCOMP-01-0124-FEDER-037296), Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-CLI/107916/2008 (http://alfa.fct.mctes.pt) and COMPETES-(FCOMP-01-0124-FEDER-008657). Financial support to V. Oliveira (SFRH/BD/46977/2008) was provided by FCT in the form of a PhD grant.

Data Accessibility

1 Sequences generated in this study can be downloaded from the NCBI Short Read Archive (Study accession: SRP035868).

CHAPTER 2

Halophyte plant colonization as a driver of the composition of bacterial communities in salt marshes chronically exposed to oil hydrocarbons

Supplementary Material

Table 2.S1. Estimators of sequence library diversity and coverage.

	BS	RH	RS
Seqs.	8493	17419	20076
OTUs	2576	2202	2648
Singletons	1388	812	947
Percent coverage (%)	83.66	95.35	95.28
Shannon diversity	5.94	5.58	6.14

BS, bulk sediment; RH, *H. portulacoides* rhizosphere; RS, *S. perennis* subsp. *perennis* rhizosphere; Seqs, Total number of sequences; OTU, Total number of Operational taxonomic unit; Singletons, Number of OTUs with one sequence; Good's estimator of coverage was calculated using the formula: (1 - (singletons/sequences)) * 100

Chapter 3

CHAPTER 3

Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria

Vanessa Oliveira, Newton C.M. Gomes, Adelaide Almeida, Artur M. S. Silva, Mário M. Q. Simões, Kornelia Smalla & Ângela Cunha

Molecular Ecology (2014) 23: 1392-1404

Abstract: Salt marsh sediments are sinks for various anthropogenic contaminants, giving rise to significant environmental concern. The process of salt marsh plant survival in such environment is very intriguing and at the same time poorly understood. Plant-microbe interactions may play a key role in the process of environment and *in planta* detoxification. In this study, a combination of culture-dependent and -independent molecular approaches [enrichment cultures, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), DNA sequencing] were used to investigate the effect of oil hydrocarbons (OH) contamination on the structure and function [polycyclic aromatic hydrocarbon (PAH) dioxygenase genes] of endophytic bacterial communities of salt marsh plant species (*Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*) in the estuarine system Ria de Aveiro (Portugal). Pseudomonads dominated the cultivable fraction of the endophytic communities in the enrichment cultures. In a set of fifty isolates tested, nine were positive for genes encoding for PAH dioxygenases (*nahAc*) and four were positive for plasmid carrying genes encoding PAH degradation enzymes (*nahA*c). Interestingly, these plasmids were only detected in isolates from most severely OH polluted sites. The results revealed site-specific effects on endophytic communities, related to the level of OH contamination in the sediment, and plant-species-specific "imprints" in community structure and in genes encoding for PAH dioxygenases. These results suggest a potential ecological role of bacterial plant symbiosis in the process of plant colonization in urban estuarine areas exposed to OH contamination.

Keywords: endophytic; salt marsh plants, polycyclic aromatic hydrocarbon, PAH degrading genes

Introduction

Salt marshes are highly productive intertidal environments that serve as nursery grounds for many commercially and economically important species. Because of their physical and biological characteristics, salt marshes are considered particularly susceptible to anthropogenic inputs of petroleum hydrocarbons (PH) (Carman *et al*., 1996; Watts *et al*., 2006; Martins *et al*. 2008). The sediment contamination with PH, especially lower molecular weight aromatic hydrocarbons, can interfere with the development of salt marsh plants (Watts *et al*., 2006; Watts *et al*., 2008). Due to the constant input of different pollutants to salt marshes located in urban areas, restoration approaches based on natural recovery processes may take decades. However, the use of plants for bioremediation (phytoremediation) associated to indigenous degrading microorganisms for removal or sequestration of pollutants has been intensively studied (Phillips *et al*., 2008; Germaine *et al*., 2009; Afzal *et al*., 2011; Khan *et al*., 2013). This approach may accelerate the process of environmental recovery and diminish the costs associated with more invasive technologies.

Several studies show that plant roots harbour a diverse microbial population in their rhizo- and endosphere that are enriched in important catabolic genotypes for hydrocarbons degradation (Gomes *et al*., 2010a; Yousaf *et al*., 2010b). These degrading bacteria may have a potential for detoxification of the sediment surrounding the roots (Geiselbrecht *et al*., 1996; Daane *et al*., 2001; Phillips *et al*., 2006; Watts *et al*., 2008; Yousaf *et al*., 2010b) or, in the case of endophytes, may be important for *in planta* hydrocarbons degradation and detoxification (Siciliano *et al*., 2001; Phillips *et al*., 2008; Yousaf *et al*., 2010b). In this scenario, endophytes may empower the plant with the biological detoxification resources required for enhanced survival in contaminated environments.

Endophytic bacteria are described as nonpathogenic bacteria colonizing internal plant tissues, without causing symptoms of disease (Taghavi *et al*., 2005; Lodewyckx *et al*., 2002). Roots are the major entry of endophytic bacteria into the plant system, presenting higher bacterial densities than other parts of the plant (Lodewyckx *et al*., 2002). Therefore, factors that are determinant in the structure of rhizosphere communities, such as soil properties (Marschner *et al*., 2001; Jung *et al*., 2008; Afzal *et al*., 2011) or plant specific factors (Grayston *et al*., 1996; Marschner *et al*., 2001;

Siciliano *et al*., 2001), are also likely to influence the diversity of endophytic communities. In general, endophytic bacteria are highly diverse and ubiquitous in most plant species (monocotyledonous and dicotyledonous) and the most common genera found are *Pseudomonas, Burkholderia, Bacillus*, and *Azospirillum* (Lodewyckx *et al*., 2002). The capacities of endophytic bacteria to act as plant growth promoters or enhance plant resistance to pathogens are known (Compant *et al*., 2005; Zachow *et al*., 2008). Recently, studies on the structural and functional diversity of endophytic hydrocarbon degraders from different impacted environments and different types of plants (essentially plants used for hay and green manure) show distinct degradative potential for hydrocarbon pollutants (Phillips *et al*., 2008; Yousaf *et al*., 2010b). In fact, the presence of bacteria carrying genes or catabolic plasmids with genes encoding PH metabolism *in planta* may contribute to the process of plant and soil (or sediment) PH detoxification in contaminated areas. The presence of PH degradative genes on mobile genetic elements, such as plasmids, has been pointed out as an important spreading mechanism for PH catabolic abilities among bacteria in contaminated areas. Horizontal gene transfer of PH degrading genes in contaminated areas has been reported, thereby enabling the hosts with oil hydrocarbon degrading capacities and contributing to microbial community acclimation to environmental pollutants (Herrick *et al*., 1997; Ma *et al*., 2006). A better understanding of the endophytic communities of salt marsh plants in contaminated coastal ecosystems may contribute fundamental information to the development of new approaches based on plant-microbe association for restoration of impacted areas.

The rhizosphere effect in the process of PH degradation has been progressively recognized. However, only very few studies have focused on the rhizosphere of salt marsh vegetation (Daane *et al*., 2001; Mucha *et al*., 2011) and practically none on the associated endophytic bacterial communities. Moreover, sediment properties, such as hydrocarbon contamination, organic matter content, and texture, are important drivers of bacterial community composition in coastal environments (Langworthy *et al*., 1998; Gomes *et al*., 2008; Liang *et al*., 2011; Wang & Tam 2012; Gomes *et al*., 2013). However, there is a lack of studies on the influence of these parameters on the structure and function of the endophytic bacterial communities.

In this study, culture-dependent and culture-independent approaches were used to investigate the effects of PH contamination on the structure (cultivation and 16S rRNA gene community fingerprint analyses) and function (plasmids and PAH dioxygenase genes) of endophytic bacterial communities from salt marsh plant species (*Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*) in a temperate estuarine system (Ria de Aveiro, Portugal). Sediment properties were also analysed to obtain more complete characterization of the sampling sites and evaluate their potential effect on the structure of the bacterial endophytic community. This work also aimed to provide evidences that naturally occurring endophytic bacteria may have potential to contribute for *in planta* detoxification of OH compounds and to plant colonization in OH polluted urban estuarine areas.

Material and Methods

Experimental site and sampling

The salt marshes investigated in this study are located in Ria de Aveiro-Portugal, a shallow estuary-coastal lagoon system on the northwest cost of Portugal (40.7° N, 8.7° W), consisting of a complex network of channels with extensive intertidal zones (Dias *et al*., 1999). Plant samples were collected at low tide in four salt marshes: site A, 40˚35´52.64´´N, 8˚45´.0071´´W; site B, 40˚38´27.42´´N, 8˚44´15.42´´W; site C, 40˚37´32.18´´N, 8˚44´09.12´´W; site D, 40˚37´18.90´´N, 8˚39´46.28´´W (Fig. 31). From each site, four composite samples of two widely distributed salt marsh plant species (*Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*) were stored separately in sterile plastic bags. These two species were chosen for their wide

Fig. 3.1 Ria de Aveiro (Portugal) with the location of sampling stations (A, B, C or D).

distribution and ecological importance and for being two of the most common species in the study area. The endophytic community was recovered from fresh roots. Root material was vigorously washed in distilled water (5 min) and root surface was disinfected by sequential washing with 95% ethanol and 1% sodium hypochlorite supplemented with one droplet Tween 80, and rinsed three times in sterile distilled water (Barac *et al.*, 2004). A 100 µL sample of final rinsing water was spread-plated on 869 medium (Mergeay *et al*., 1985) to check the efficiency of disinfection.

Sediment properties

The pH value of bulk sediments was measured in a sediment: water suspension [1:5 (w/v)] (Faoun 1984), using a pre-calibrated pH meter (Orion Model 290A). Organic matter (OM) content was analyzed as percentage of weight loss by ignition at 450 °C, for 8 h (SPAC 2000). Sediment grain size was analyzed by wet and dry sieving (Quintino *et al*., 1989).

Freeze-dried sediment samples were homogenized and analyzed for aliphatic and aromatic hydrocarbons after a Soxhlet extraction followed by GC-MS analysis. For assessing the efficiency of recovery, 5 g sub-samples of sediment were spiked with 25 µL (2 µg mL^{-1}) deuterated surrogate standards (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12} (Supelco) and extracted for 24 h in a Soxhlet apparatus with 150 mL of dichloromethane. Sample extracts were concentrated by rotary evaporation up to a volume of about 2-3 mL, and solvent changed to 10 mL of hexane, which was further reduced to approximately 1-2 mL. Hydrocarbons concentrated in hexane extracts were separated using a deactivated 2:1 alumina/silica gel column. Elution was performed using 15 mL of hexane to yield the first fraction (aliphatic hydrocarbons), followed by 30 mL of dichloromethane:hexane (1:1). Sample volume was reduced to 1 mL on a rotary evaporator, evaporated under nitrogen gas to 0.2 mL, transferred to a GC vial, and 25 µL of the internal standard, hexamethylbenzene (2 mg mL^{-1}) were added. GC-MS analyses were carried using an Agilent Network GC system, namely an Agilent 6890 gas chromatograph equipped with a mass selective detector (MSD 5973) with electron impact ionization (EI) operating at 70 eV (Agilent Technologies). A VF-5MS fused silica column (30 m x 0.25 mm i.d and 0.25 µm film thickness) was used for analytes separation. The carrier gas used was helium with a

flow rate of 1.3 mL min^{-1}. Injector and transfer-line temperatures were set at 300 ºC. The ion source was maintained at 230 ºC. A 1 µL splitless injection volume was delivered with a 10 µL Hamilton syringe. The initial column temperature was maintained at 60 ºC for 1 min, ramped to 200 ºC at a rate of 10 ºC min^{-1}; a second ramp of 5 ºC min^{-1} raised the column temperature to 300 ºC, which was kept for 8 min. For aromatic hydrocarbons the selected ion monitoring (SIM) acquisition mode was used. The full scan acquisition mode (in a range between *m/z* 20 and *m/z* 500) was used to detect aliphatic hydrocarbons, and for quantification two internal standards were used: undecane ($C_{11}H_{24}$) and tetracosane ($C_{24}H_{50}$).

Endophytic communities

DNA extraction

Extraction of total DNA from the endophytic communities (TC-DNA) was performed using a bead-beating protocol previously outlined in the study by Phillips *et al*., 2006. About 2 g of surface disinfected root material was macerated in liquid nitrogen. The cell lysis was achieved by a combination of bead-beating, proteinase K (20 mg mL^{-1}) and sodium dodecyl sulfate (20%). Cellular debris and proteins were precipitated with aqueous 7.5 M ammonium acetate. DNA was subsequently precipitated with isopropanol, washed with 70% cold ethanol and re-suspended in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The endophytic TC-DNA was purified with the GeneClean Spin Kit (MP Biomedicals).

DGGE analysis

The structural diversity of the endophytic communities was assessed by DGGE fingerprint analysis of TC-DNA of 16S rRNA gene fragments, after a nested-PCR approach (Gomes *et al*., 2008). First, 16S rRNA gene fragments were amplified using the primers U27F and 1492R (Table 1). Reaction mixtures (25 µL) contained 12.5 µl DreamTaq™ PCR Master Mix (Fisher Scientific), 0.1 µM of each primer, 80 µg mL^{-1} bovine serum albumin (BSA) and 1 µL of template DNA. The amplification conditions were as follows: initial denaturation (94 ºC for 5 min); 25 cycles of denaturation (94 ºC for 45 s), annealing (56 ºC for 45 s), and extension (72 ºC for 1.5 min), and a final extension (72 ºC for 10 min). The amplicons obtained from the first PCR were used as a

template for a second PCR with bacterial DGGE primers 984F-GC and 1378R (Table 1). The PCR reaction mixtures (25 µL) consisted of 12.5 µl DreamTaq™ PCR Master Mix (Fisher Scientific), 0.1 µM of each primer, 1 % (v/v) dimethyl sulfoxide (DMSO) and 1 µL of template DNA. After 4 min of denaturation at 94 ºC, 30 thermal cycles of 1 min at 95 °C, 1 min at 53 °C, and 1.5 min at 72 °C, the PCR was finished by an extension step at 72 °C for 7 min. Five µL of PCR products were analyzed by electrophoresis on a 1% agarose gel and stained with GelRed (Biotium).

The DGGE of the amplified 16S rRNA gene fragments was performed using DCode System (Universal Mutation Detection System, Bio-Rad). PCR products containing approximately equal amounts of DNA were loaded onto 6-10 % (w/v) polyacrylamide gel in 1 x TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA; pH 8.0). The 6-10 % polyacrylamide gel was made with a denaturing gradient ranging from 40 to 58 %. Electrophoresis was performed for 16 h at 80 V at 60 °C in 1 x TAE buffer. Following electrophoresis, the gels were silver-stained according to Heuer *et al*., (2001).

Detection of PAH degrading genes

The presence of genes encoding the large subunit of naphthalene-1,2-dioxygenase (*nahAc*) and alpha subunit of the PAH ring-hydroxylating dioxygenases genes, both related to PAH degradation, was detected by PCR using the primers and reaction conditions described by Wilson *et al*., (1999) and Cébron *et al*., (2008), respectively (Table 3.1).

Determination of gene copy number of PAH ring-hydroxylating dioxygenase genes of Gram-negative bacteria

Quantification of PAH ring-hydroxylating dioxygenase of Gram-negative bacteria was carried out using the primers and probe previously described by Cébron *et al*., (2008) by real-time PCR (Applied Biosystems StepOne™ Real-Time PCR System). Amplification was performed in 20 µL reaction volume containing 11 µL SYBR® Green PCR Master Mix (Applied Biosystems), 0.2 µM of each primer (IBA GmbH), 0.55 µL of DMSO, 100 µg mL^{-1} of BSA and 1 µL of template TC-DNA. The temperature profile was 95 ºC for 10 min followed by 45 cycles at 95 ºC for 30 s, 57 ºC for 30 s, 72 ºC for 30 s and a data collection step of 10 s at 80 ºC. The final step

consisted of 72 ºC for 7 min. A final melt curve analysis, from 6 ºC below amplification temperature, to 95 ºC, concluded the cycling program. Templates to generate standard curves were serial dilutions of amplicon of *Pseudomonas putida* pNF149 (10^8 to 10^2). The samples were 10 times diluted prior to amplification to avoid problems with inhibitors. DNA samples were used at a concentration of 4-11 ng μL^{-1}.

Table 3.1 Primers and conditions used for PCR amplification.

Primer	Primer sequence (5´to 3´)	Annealing temperature (ºC)	Primer (µM)	Fragment size (bp)	Reference
Universal 16S rRNA					
U27F	AGA GTT TGA TCC TGG CTC AG	56	0.25	ca. 1450	(Weisburg *et al*., 1991)
1492R	GGT TAC CTT GTT ACG ACT T				
968F-GC*	AAC GCG AAG AAC CTT AC	53	0.25	ca. 473	(Heuer *et al.*, 1997)
1378R	CGG TGT GTA CAA GGC CCG GGA ACG				
PAH degrading genes					
Protocol 1: Naphthalene dioxygenase (nahAc)					
Ac114F	CTG GC(T/A)(T/A)TT (T/C)CT CAC (T/C)CA T	56.5	0.2	ca. 482	(Wilson *et al*., 1999)
Ac596R	C(G/A)G GTG (C/T)CT TCC AGT TG				
PAH ring-hydroxylating dioxigenase (PAH-RHDα)					
Protocol 2					
PAH-RHDα GNF	GAG ATG CAT ACC ACG TKG GTT GGA	57	0.2	ca. 306	(Cébron *et al*., 2008)
PAH-RHDα GNR	AGC TGT TGT TCG GGA AGA YWG TGC MGT T				
Protocol 3					
PAH-RHDα GPF	CGG CGC CGA CAA YTT YGT NGG	54	0.2	ca. 292	(Cébron *et al*., 2008)
PAH-RHDα GPR	GGG GAA CAG GGT GCC RTG DAT RAA				
BHR plasmids					
IncP-1(trfA genes)					
trfA 733f F	TTC ACS TTC TAC GAG MTK TGC CAG GAC	60	1.5	ca. 281	(Bahl *et al.*, 2009)
trfA 1013 R	GWC CAG CTT GCG GTA CTT CTC CCA				
trfA g F	TTC ACT TTT TAC GAG CTT TGC AGC GAC				
trfA g R	GTC AGC TCG CGG TAC TTC TCC CA				
trfA d F	TTC ACG TTC TAC GAG CTT TGC ACA GAC				
trfA d R	GAC AGC TCG CGG TAC TTT TCC CA				
IncP-7 (rep genes)					
P7 repB F	GCA CAA ACG GTC GTC AG	54	0.25	ca. 524	(Izmalkova *et al.*, 2005)
P7 repA R	CCC TAT CTC ACG ATG CTG TA				
IncP-9 (Ori-rep genes)					Unpublished Flocco/Ding
ori 69 F	GAG GGT TTG GAG ATC AT(AT) AGA	53	0.2	ca. 610	
ori 679 R	GGT CTG TAT CCA GTT (AG)TG CTT				

*Preceded by a GC clamp to DGGE, CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G

Endophytic isolates

Enrichment and isolation of PH-degrading bacteria

Endophytic extracts were obtained by root maceration (0.5 g) in 10 mL of 10 mM $MgSO_4.7H_2O$ using a Stomacher 400 (Seward Ltd). The enrichment consisted of a

series of batch cultures containing two different carbon sources: 2-methylnaphthalene and diesel oil mixture (98 octane gasoline with 2 % engine oil). The first enrichment consisted of inoculation with 1 mL of endophytic extract to 50 mL of sterile mineral medium (MM) (Ma *et al*. 2006) supplemented with 10 mg of 2-methylnaphthalene crystals or with 1% filter-sterilized diesel oil (0.2 μm, PTFE membranes). Cycloheximide (100 mg/L) was added to the selective media to suppress fungal growth. Control flasks without root extracts were included. The flasks were covered with non-absorbent cotton wool and incubated at room temperature in the dark with agitation (100 rpm) for 7 days. Two more selection rounds were conducted by sub-culturing 1 mL from the previous culture in 50 mL of mineral medium with the corresponding carbon source. Aliquots of each selective culture were stored at -80 ºC in 1 mL of glucose broth (Fluka) with 25 % glycerol, for molecular studies.

From each selective culture, 100 µL replicate aliquots were spread-plated in two different media: R2A and King B (Merck). Isolated colonies were selected based on morphology and color and purified by successive streak-plating on R2A and King B medium. After purity confirmation, selected colonies were transferred to LB Medium with 15 % glycerol and frozen at - 20 °C until processing.

DNA extraction

Extraction of genomic DNA from endophytic isolates was performed after cell lysis with the Qiagen genomic DNA extraction kit (Qiagen), in combination with Fermentas DNA Extraction Kit (Fermentas).

Characterization and identification of endophytic strains

Endophytic strains were grouped by the similarity of BOX-PCR genomic fingerprints using the BOX_A1R primer (Martin *et al*., 1992). For strains with distinct fingerprints, the 16S rRNA gene was amplified using PCR universal bacterial primers U27 and 1492R (Weisburg *et al*., 1991) and sequenced. The partial 16S rRNA sequences were compared with different sequences available in the GenBank database using BLAST-N (Basic Local Alignment Search Tool).

Detection of genes encoding for PAH degradation

The presence of *nahAc* and PAH ring-hydroxylating dioxygenase genes was detected by PCR using the primers and reaction conditions described by Wilson *et al*. (1999) and Cébron *et al*., (2008), respectively (Table 3.1), as previously described for community DNA.

PCR-detection of broad host range (BHR) plasmids

A PCR approach was used for detection of BHR plasmid-specific sequences in DNA extracted from isolated endophytic strains. The amplifications of plasmid-specific sequences of IncP-1 (trfA gene), IncP-7 (rep gene) and IncP-9 (oriV-rep genes) were performed with the primers and conditions summarized in Table 3.1. The positive PCR amplicons were confirmed by Southern-blot hybridization (SBH) on Hybond N nylon membranes (Amersham Hybond-N; GE Healthcare) according to Sambrook & Russell (2001). PCR amplicons of IncP-9 were hybridized with digoxigenin-labeled mixed probe generated from typical IncP-9 plasmids. Detection of hybridization with the DIG-labeled probe was performed with a DIG luminescent detection kit (Roche Applied Science) as specified by the manufacturer, using an X-ray film (Roche Applied Science).

Plasmid-DNA extraction and characterization

Plasmids were obtained by alkaline extraction according to the adaptation of the procedure by Birnboim & Doly (1979) described by Smalla *et al*., (2000). For plasmid characterization, extracted DNA was digested for 4 h at 37 ºC with a combination of the restriction enzymes PstI and Bst11071 (Fisher Scientific). Restriction fragments were separated on a 0.8 % agarose gel for 2 h at 50 V and stained with GelRed (Biotium).

Detection of genes encoding for PAH degradation enzymes in plasmidic DNA

The presence of specific catabolic genes encoding enzymes involved in hydrocarbon degradation in plasmids extracted from isolated strains was assessed using specific primers for the *nahAc* and PAH ring-hydroxylating dioxygenase genes (Table 3.1). A touchdown PCR programme was used for the amplification of *nahAc*-like genes, as

described by Wilson *et al.*, (1999). A 16S rDNA PCR negative control reaction was conducted to ensure the absence of bacterial chromosomal DNA.

Sequence analysis of PAH degrading genes and phylogenetic tree

Amplicons of PAH degrading genes were cleaned using GeneClean II Kit (MP Biomedicals) following the manufacturer´s instructions and sequenced at external facilities (StabVida). Sequenced genes were compared with sequences available in the GenBank® database by using the BLAST service to determine their closest relative.

Statistical analysis

The DGGE profile analysis was conducted with the software package BioNumerics v6.6 (Applied Maths, Belgium). The matrix containing both band position and intensity was processed in a spreadsheet and transformed into relative abundance. The relative abundance matrix was log10 (x+1) transformed, and a distance matrix was constructed with the Bray Curtis similarity values. Distance values were used to ordinate profiles by nonmetric multidimensional scaling (NMDS) analysis using PRIMER 6 software. Environmental variables, namely pH, organic matter, moisture, % fines and hydrocarbon concentration were fit in NMDS community composition analysis using a Pearson correlation. The distance based linear models (DistLM) was carried out to identify correlations between measured sediment parameters and the composition of endophytic community. For the initial marginal test, the AIC (Akaike Information Criterion) was selected, basing the analysis on the Bray-Curtis resemblance measure after square root transformation of the abundance data. A two-way permutational analysis of variance (perMANOVA) was used to analyze the differences in endophytic community composition and gene copy number between plant species and sampling sites.

The amino acid sequences deduced of PAH dioxygenase genes fragments were aligned to their closest relatives. A phylogenetic tree was then constructed using the Neighbor-joining algorithm applied gamma distribution to a Jones-Taylor-Thornton (JTT) model and bootstrapping (500 repetitions) analysis using the Molecular Evolutionary Genetics Analysis (MEGA5) software.

Results and Discussion

Environmental variables and endophytic community composition

The location of the sampling sites within the complex estuarine system of Ria de Aveiro influenced the physical-chemical properties of the sediment, such as organic matter content and OH concentration (Table 3.2). According to the total concentration of aromatic (16 PAHs) and aliphatic PH, site B is the least (5.86 ng gram^{-1} dry weight) and site C the most contaminated site (21.39 ng g^{-1} dw). Sites A and D showed similar levels of contamination with intermediary values (11.05 and 11.47 ng g^{-1} dw, respectively). Site C is more exposed to anthropogenic activities (recreational navigation, urban runoff and shipping activity), while site A is located in the same main channel but with lower influence of the Port of Aveiro. Site B is not exposed to direct contamination as it is located in a secondary channel, and finally, site D is also located near a secondary channel but is exposed to the influence of industrial activities.

Table 3.2. Sediment properties and concentration of hydrocarbons in four salt marshes sites of the estuarine system Ria de Aveiro (mean value ± STD, n = 3).

	Sampling sites			
	A	B	C	D
pH	7.12 ± 0.10	8.34 ± 0.20	7.49 ± 0.26	7.24 ± 0.24
Organic matter (%)	4.54 ± 1.13	2.67 ± 1.15	5.81 ± 2.08	7.20 ± 2.97
Moisture (%)	22.47 ± 5.03	17.74 ± 3.18	36.61 ± 8.18	40.10 ± 10.33
% Fines	58.06 ± 0.06	20.96 ± 0.06	81.99 ± 0.13	62.67 ± 0.03
Sediment Texture	Mud	Very fine sand	Mud	Mud
Hydrocarbons (ng g^{-1} dry weight)				
Σ Aliphatics (*n*-C_{10}-C_{32})	9.76 ± 0.17	3.79 ± 0.06	10.25 ± 0.14	9.53 ± 0.14
Σ 16 PAH	1.29 ± 0.01	2.07 ± 0.05	11.13 ± 0.14	1.94 ± 0.05
Σ Total	11.05 ± 0.11	5.86 ± 0.01	21.39 ± 0.00	11.47 ± 0.07

The DistLM analysis was carried out to identify correlations between measured sediments parameters and the composition of endophytic bacterial assemblages (DGGE profiles). Strong dependence on sediment parameters was observed in the endophytic community composition of *H. portulacoides* and *S. perennis* subsp. *perennis* plants, explaining 63% and 71% of variation, respectively. Using AIC to find the simplest combination of sediment parameters which best explained the bacterial assemblage

patterns, pH, log (Σ 16PAHs) and organic matter were identified as the best fit model, explaining 51 % of variation in *H. portulacoides* plants and 58 % of variation in *S. perennis* subsp. *perennis* plant. In agreement with our results, Gomes *et al* (2007, 2008, 2013) provided evidences that the degree of sediment contamination and especially the type of pollutants present can influence the structure of bacterial communities inhabiting estuarine sediments. Soil properties, such as pH and organic matter (Marschner *et al.*, 2001; Jung *et al.*, 2008; Afzal *et al.*, 2011), are also important drivers in the process of bacterial colonization in rhizosphere microhabitats. However, there is a lack of information about the influence of the parameters evaluated in this study on the estuarine endophytic communities.

Plant species effects on endophytic bacterial communities

The endophytic bacterial assemblage patterns inferred from DGGE analyses were ordinated using NMDS (Fig. 3.2A and 2B). With exception of sites A and C in *S. perennis*, the NMDS ordination revealed significant changes in the composition of bacterial communities from different plant species [two-way perMANOVA ($p < 0.001$)] (data not shown). Plant effects on the microbial community in surrounding sediments can be species specific (Berg & Smalla 2009). Root morphology and exudates are known drivers in the process of rhizosphere colonization by soil bacteria (Marschner *et al*., 2001; Berg & Smalla 2009; Bais *et al*., 2006) and may be expected to contribute for *in planta* colonization of endophytic bacteria. Root exudates, more specifically, may contain various molecules that are structurally related to several environmental pollutants (e.g. PAHs) with potential to enhance soil bacteria carrying catabolic pathways for aromatic compounds (Shaw & Burns 2003). A previous study (Oliveira *et al*., 2012) provided evidences that *H. portulacoides* and *S. maritima* root systems may contribute for the release of species specific volatile compounds in their root exudates.

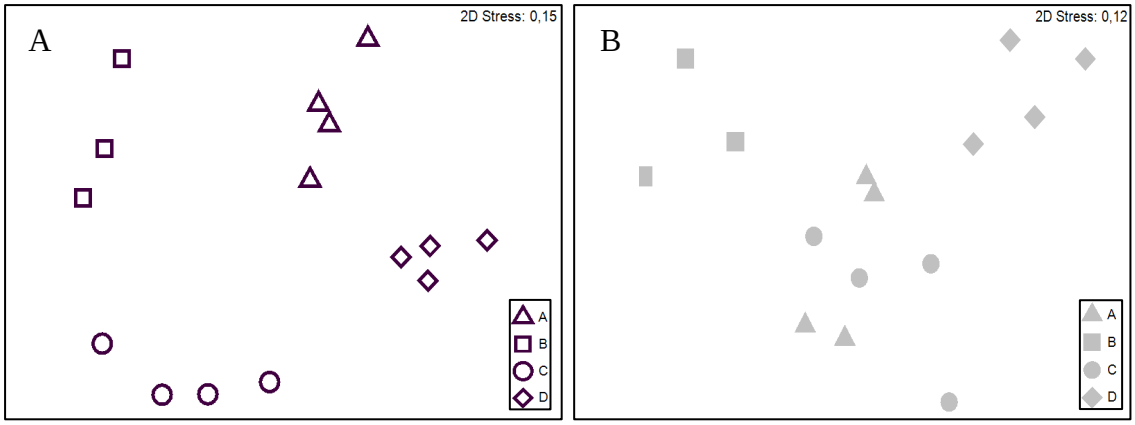

Fig. 3.2 NMDS showing the comparison between DGGE fingerprints of endophytic communities from two different plants, *Halimione portulacoides* (A) and *Sarcocornia perennis* subsp. *perennis* (B), along a gradient of hydrocarbons contamination.

PAH dioxygenase genes in total endophytic communities

Genes encoding for PAH dioxygenase were present in members of the endophytic communities found in the roots of *H. portulacoides* and *S. perennis* subsp. *perennis*, indicating a potential metabolic capacity of endophytic bacteria for degradation of PH. In this study, different sets of primers for detection of genes encoding for PAH degrading enzymes were used. The sequence analyses showed that the majority of genes detected in endophytic communities were closely related to the *nahAc* gene. Interestingly, some genotypes showed plant-species-specific association (Table 3.S1). Although catabolic genes associated with hydrocarbon degradation are considered to be widespread, some genotypes can show specific associations with some plant species (Phillips *et al*., 2008). Other studies have shown that specific catabolic genotypes are plant species dependent, although also influenced by environmental factors (Siciliano *et al*., 2001, Cébron *et al*., 2009, Ding *et al*., 2010). The results obtained in this study indicate that the abundance of genes encoding for PAH ring-hydroxylating dioxygenase in the endophytic communities tend to show differences between plant species and sampling sites (Fig. 3.3). Gene copy numbers were highest in plants located in more contaminated sites (C and D). However, perMANOVA analysis did not confirm significant differences between sites. A similar plant/contaminant-dependent selection of different catabolic genotypes in endophytic populations was observed in *Scirpus*

pungens and *Festuca arundinacea* after experimental soil contamination with nitroaromatics and petroleum (Siciliano *et al.*, 2001).

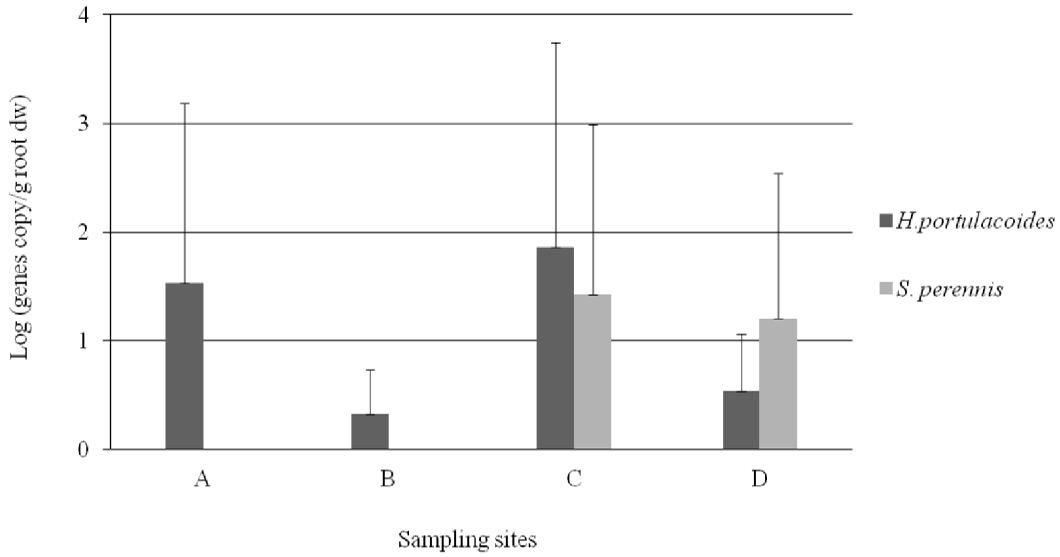

Fig. 3.3 Quantification of PAH-RDHα GN gene copy number contained in endophytic community in a gradient of hydrocarbons contamination (mean value ± STD, n = 4).

The preponderance of the factors related either to plant attributes or to sediment properties may be difficult to decide. However, a study about the diversity of *ndo* genes in rhizosphere of two vascular plants growing in Antarctic soils revealed that the selection of *ndo* genotypes was more related to the contamination than to the influence of plants (Flocco *et al.*, 2009). Also, the effects are most likely interdependent. It has been demonstrated that plant growth can be affected by soil characteristics (soil type, particle sizes and organic matter content) which also influence microbial colonization and activity, and subsequently the efficiency of contaminant degradation (Afzal *et al.*, 2011).

Table 3.3 Endophytic bacterial isolates from enrichment cultures of *H. portulacoides* and *S. perennis* subsp. *perennis* roots.

Sample ID	Sequence acession nº.[a]	16S rRNA Identity	% Similarity	Acession nº.[b]
Halimione portulacoides				
Naphthalene				
1	KF135227	*Pseudomonas* sp.	99	JX047434
3	KF135228	*Sphingobium yanoikuyae*	100	JX122496
5	KF135229	*Pseudomonas* sp.	99	EF627998
6	KF135230	Moraxellaceae	100	JF947030
7	KF135231	*Pseudomonas rhodesiae*	99	JX994152
8	KF135232	*Micrococcus* sp.	99	JQ396588
9	KF135233	*Sphingobium* sp.	99	JQ433940
9a	KF135234	*Pseudomonas* sp.	100	EF628000
11	KF135235	*Pseudomonas* sp.	100	AF411853
13	KF135236	*Pseudomonas* sp.	99	HF679142
Diesel oil				
36	KF135237	*Pseudomonas rhodesiae*	100	JX994152
40	KF135238	*Pantoea dispersa*	99	JN835497
42	KF135239	*Pseudomonas* sp.	100	AB772943
Sarcocornia perennis subsp. *perennis*				
Naphthalene				
15	KF135240	*Microbacterium* sp.	99	JN942146
17	KF135241	*Pseudomonas* sp.	99	EU306338
18	KF135242	*Pseudomonas putida*	100	JQ824856
19	KF135243	*Pseudomonas* sp.	100	GU966669
21	KF135244	*Acinetobacter* sp.	97	HM755663
24	KF135245	*Pseudomonas* sp.	100	AY332207
25	KF135246	*Ochrobactrum* sp.	99	JQ821380
26	KF135247	*Pseudomonas* sp.	100	JN033360
27	KF135248	*Microbacterium* sp.	100	AB733571
29	KF135249	*Pseudomonas stutzeri*	99	EU167940
31	KF135250	*Pseudomonas* sp.	100	EF628000
35	KF135251	*Pseudomonas putida*	100	JQ619028
Diesel oil				
49	KF135252	*Pseudomonas* sp.	100	JN033360

[a] GenBank sequence accession numbers of the respective isolate
[b] GenBank sequence accession numbers of the most related bacterial sequence

Endophytic bacteria enriched in selective cultures

Fifty putative hydrocarbonoclastic endophytic bacterial strains were isolated from selective cultures. After BOX-PCR typing, twenty-six isolates were selected as clone representatives. Their 16S rRNA gene sequences showed 98-100% similarity to known genes deposited in the GenBank database and closely related with organisms obtained from polluted sites (Table 3.3). Sequence analysis of these strains revealed a great predominance of Gram-negative bacteria, particularly *Pseudomonas* sp. Only few endophytic Gram-positive bacteria were isolated (Table 3.3). The predominance of *Gammaproteobacteria*, mainly *Pseudomonas* sp., is consistent with previous observations of this group associated to the degradation of hydrocarbons (Ma *et al*., 2006) in rhizospheres as well as in root endophytic communities (Phillips et al. 2008; Taghavi et al., 2009; Yousaf et al., 2010b). *Sphingobium*, *Acinetobacter*, *Microbacterium*, *Micrococcus* and *Ochrobactrum* were also found among the isolates. In a similar work, twenty-one PAH degrading bacteria strains isolated from Antarctic soils were identified as *Pseudomonas* and associated to PAH degradation (Ma *et al*., 2006). *Sphingobium* sp. is reported as a common PAH degrader in soils (Cunliffe & Kertesz 2006) and *Acinetobacter* sp., in association with other bacteria, is also involved in the degradation of a variety of PAHs (Yu *et al*., 2005). Crude-oil-degrading strains of *Microbacterium* are also described in the literature (Schippers *et al*., 2005), and two *Micrococcus* strains capable of growing on a mixture of naphthalene and phenanthrene were isolated from soil near an oil refinery (Ashok *et al*., 1995). *Ochrobactrum* is also reported as able to grow on a variety of PAHs, namely phenanthrene, pyrene, fluoranthene and benzo[*a*]pyrene (Wu *et al*., 2009).

PAH genes and BHR plasmids in endophytic isolates

In this work, three sets of primer were used to target genes encoding for PAH degradation in endophytic strains (Table 3.S2). These genes were only detected in few isolates and mainly in cultures obtained from naphthalene enrichments. In general, the isolation of microorganisms capable of growing on naphthalene is widely documented (Widada *et al*., 2002). In selective cultures containing diesel oil as carbon source, genes encoding for PAH degradation were only detected on three *Pseudomonas* isolates. This may be related to the fact that diesel oil represents a more diverse carbon source,

containing a small fraction of naphthalene and a range of aromatic and aliphatic hydrocarbons that may be used by different and specialized metabolic pathways (Ciric *et al*., 2010).

The use of culturable fractions of the endophytic communities as retrieved from the selective cultures might also explain the differences in gene prevalence. In a study conducted in mangrove microniches, the composition of the original community of the microniches determined the structural and functional diversity of the PAH degrading enrichments (Gomes *et al*., 2010a).

The phylogenetic tree of the predicted amino acid sequences of gene fragments encoding for PAH dioxygenase from endophytic isolates shows a close phylogenetic relationship with the large α-subunit of naphthalene dioxygenase belonging to the *nahAc* genotypes described for *Pseudomonas* species and/or plasmids associated with this genus (Fig. 3.4, 3.5). In Figure 3.4, the amino acid sequence retrieved from the three strains (protocol 1) is closely related to archetypal *nahAc* gene sequences of *Pseudomonas*-associated plasmids (sequences identity within the group 73%).

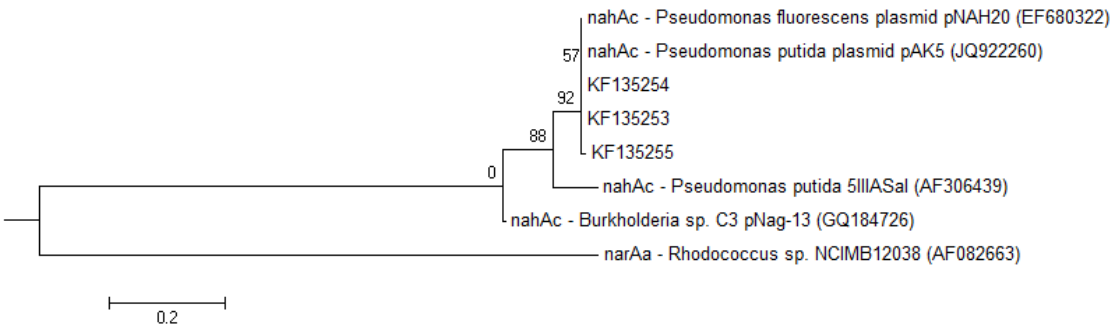

Fig. 3.4 Phylogenetic relations of amino acid sequences of genes encoding PAH degradation enzymes detected in bacterial isolates using protocol 1. The sequences were aligned with related sequences retrieved from GenBank.

The protocol 2 detected genes encoding for PAH degradation closely related to the *nahAc* gene previously described for *Pseudomonas* sp. LCY18 and *Pseudomonas* associated plasmid (Fig. 3.5). The occurrence of *nah*-like genotypes carried by PAH degrading bacteria have been reported in isolates from enrichment cultures with naphthalene or phenanthrene as carbon source (Ma *et al*., 2006) and in the rhizosphere

of two plants growing in Maritime Antarctic (Flocco *et al*., 2009). A succession of different genes encoding PAH dioxygenase was found in PAH-contaminated environments, and a dominance of *nah*-like genotypes occurred after enrichment with naphthalene (Chadhain *et al*., 2006). Moreover, our results indicate that different genes encoding for PAH degradation occur when exposed to different PAHs in culture medium. The results obtained in this work underline the potential environmental importance of *nah* genotypes in the process of PAH degradation by bacterial endophytes in salt marsh plants.

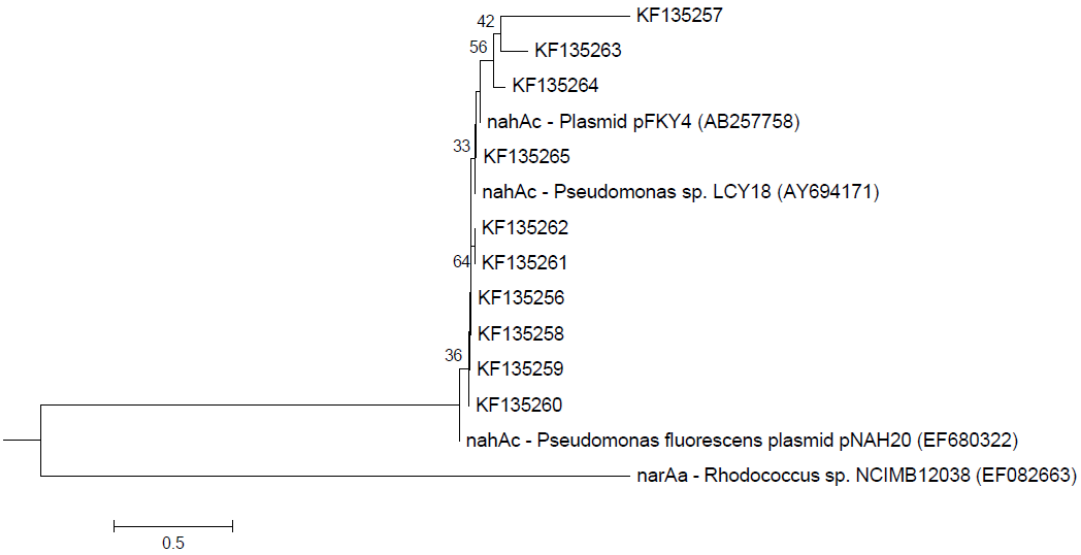

Fig. 3.5 Phylogenetic relations of amino acid sequences of genes encoding PAH degradation enzymes detected in bacterial isolates using the set of primers of protocol 2. The sequences were aligned with related sequences retrieved from GenBank.

Horizontal gene transfer mediated by catabolic plasmids plays an important role in the adaptation of microbial communities to chemical stress in polluted environments (Dennis 2005). For this reason, in this study, the potential role of BHR plasmids (IncP-1, IncP-7 and IncP-9) in the process of horizontal transfer of genes encoding PAH dioxygenase was investigated (Table 3.S2). A total of 18 isolates, all from the selective cultures containing naphthalene, carried IncP-9 plasmids (Table 3.S2). However, IncP-1 and IncP-7 plasmids were not detected. IncP-9 catabolic plasmids belong to a known group of self-transmissible plasmids that often encode enzymes involved in the process

of PH degradation. In this study, sequence fragments obtained from genes encoding for PAH dioxygenase in plasmids exhibited high similarity with the *nahAc* gene of plasmid FKY4, a IncP-9 self-transmissible plasmid able to transfer the ability to degrade naphthalene to other bacterial cells (Ono *et al*., 2007) (Fig. 3.5). Our results indicate that this plasmid may be involved in the spread of *nah* genotypes between salt marsh plants in the estuary studied.

Conclusions

This study assessed the potential effects of petrogenic hydrocarbon contamination on the structural and functional diversity of endophytic bacteria in two salt marsh plant species. The results showed that plant species and sediment properties are likely interacting in the modulation of the endophytic bacterial communities and in their genetic determinants for OH degradation, like genes and plasmids. Furthermore, plant-species-specific "imprints" were confirmed in the composition of genes encoding for PAH degradation. Based on the phylogenetic analysis, the culturable fraction of endophytic degraders was essentially close related to known PAH-degrading *Pseudomonas* species. The prevalence of *nah* genotypes and the detection of catabolic plasmids in salt marsh plants indicate that horizontal gene transfer can drive the spread of *nah* genes in these habitats and probably contribute to the process of bacterial endophytic adaptation and *in planta* OH decontamination. Moreover, *nah* genes are commonly associated with hydrocarbon degrading species belonging to the genus *Pseudomonas*. Overall, the results suggest that the adaptation to OH contamination goes beyond the rhizosphere communities and extends to endophytic bacteria tightening the relations established between plants and microbes in response to OH contamination.

Acknowledgements

This work had financial support from CESAM research unit (PEst-C/MAR/LA0017/2011), QOPNA research unit (project PEst-C/QUI/UI0062/2011), Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-CLI/107916/2008 (http://alfa.fct.mctes.pt) and COMPETES-(FCOMP-01-0124-FEDER-008657). Financial support to V. Oliveira (SFRH/BD/46977/2008) was provided by FCT in the form of a PhD grant.

Data Accessibility

1 The 16S rRNA sequences for the OH-degrading isolates have been deposited in GenBank under accession numbers KF135227 to KF135252.

2 The PAH degrading genes sequences obtained were deposited in the GenBank database under the accession numbers KF135253 to KF135265.

CHAPTER 3

Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria

Supplementary Material

Table 3.S1 Detection of genes encoding PAH degradation enzymes on endophytic community from roots in a gradient of hydrocarbon contamination. -, no detection in all replicates; +, detection in one replicate; ++, detection in two replicates; +++, detection in three replicates (n = 4).

	Sampling sites			
	A	B	C	D
Halimione portulacoides				
PAH degrading genes				
Protocol 1[a]	-	-	-	-
Protocol 2[b]	++	+++	++	++
Protocol 3[c]	-	-	-	-
Sarcocornia perennis subsp. *perennis*				
PAH degrading genes				
Protocol 1[a]	+++	++	-	++
Protocol 2[b]	-	-	++	++
Protocol 3[c]	-	-	-	-

[a] Protocol 1: Primer set Ac114F and Ac596R
[b] Protocol 2: Primer set PAH-RHDα GN F and PAH-RHDα GN R
[c] Protocol 3: Primer set PAH-RHDα GP F and PAH-RHDα GP R

Table 3.S2 PCR and SBH analysis of genes encoding PAH degradation enzymes involved in hydrocarbon degradation and plasmid groups of endophytic isolates. The signal + represents the number of isolates where genes were detected. - = hybridization and (-) = weak hybridization.

	Naphthalene				Diesel oil			
	Sampling sites							
	A	B	C	D	A	B	C	D
H. portulacoides								
PAH degrading genes								
Protocol 1[a]				+				
Protocol 2[b]			+	+	+			
Protocol 3[c]								
BHR plasmids								
IncP-1								
IncP-7								
IncP-9	++	+	+	+++++				
IncP-9[d]	- (-)	(-)	(-)	(-----)				
S. perennis subsp. *perennis*								
PAH degrading genes								
Protocol 1[a]			+	+				
Protocol 2[b]		+		+		+	+	
Protocol 3[c]								
BHR plasmids								
IncP-1								
IncP-7								
IncP-9	++++	+++	++					
IncP-9[d]	-- (--)	--- (-)	- (-)					

[a] Protocol 1: Primer set Ac114F and Ac596R
[b] Protocol 2: Primer set PAH-RHDα GN F and PAH-RHDα GN R
[c] Protocol 3: Primer set PAH-RHDα GP F and PAH-RHDα GP R
[d] Isolates which hybridized with IncP-9 probe

Chapter 4

CHAPTER 4

Evaluation of plant-microbe interactions in 2-methylnaphthalene contaminated sediments

Abstract: The interaction between plants and their associated indigenous degrading microorganisms (rhizo- and endosphere) have been explored as a process for the removal or sequestration of pollutants. In this study, a factorial microcosm approach was used to investigate the response of the salt marsh halophyte *Halimione portulacoides* to contamination with 2-methylnaphthalene. The effect of inoculation with indigenous endophytic hydrocarbon degrading bacterium *Pseudomonas* sp. was also assessed. During the experiments, plant photosynthetic performance was monitored by Pulse Amplitude Modulated (PAM) fluorometry, the structural diversity and function of bacterial communities were assessed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA gene sequences, and the detection of polycyclic aromatic hydrocarbon (PAH) dioxygenase genes was attempted. The clearance of the added hydrocarbon was evaluated by chemical analysis of the sediments.

The endophytic and rhizosphere communities exhibited different responses to inoculation and/or to 2-methylnaphthalene amendment. The inoculation had a positive impact in plant photophysiology but it did not cause a significant change in the plant health responses to hydrocarbon exposure. The addition of 2-methylnaphthalene increased the frequency of genes encoding for the large subunit of naphthalene-1,2-dioxygenase (*nahAc*) in sediment bacteria (with and without inoculation).

These results do not demonstrate a significant effect of the inoculation with an endophytic hydrocarbon-degrading bacteria on the overall hydrocarbon degradation potential of the salt marsh halophyte *Halimione portulacoides,* under the moderate PAH concentrations of the experiment. However, the effect of the inoculant on the structure of the indigenous endophytic community indicates that the strain used in this study is a good plant colonizer and may be regarded as promising candidate for microbe-assisted phytoremediation approaches.

Keywords: endophytic; *H. portulacoides*, polycyclic aromatic hydrocarbon, phytoremediation

Introduction

Estuarine areas are very susceptive to contamination by oil hydrocarbons, mainly because they are often located in the vicinity of urban and industrialize areas. Petroleum-derived, such as, polycyclic aromatic hydrocarbons (PAHs), are of particular importance due to their ubiquity, toxicity and persistence (Haritash & Kaushik, 2009). Removal techniques are being applied to water and sediment in the attempt to restore environmental quality, with a preponderance of in situ methods which are more efficient and cost-effective (Alkorta & Garbisu, 2001).

Phytoremediation as the use of vegetation to enhance degradation and removal of contaminants in soil, sediments and groundwater has emerged as the best natural technology alternative (Escalante-Espinosa *et al.*, 2005, Moreira *et al.*, 2011). Successful application rely on a variety of factors, such as the type and quantity of contaminant, performance of particular plant species or physical and chemical characteristics of the contaminated area (EPA, 200, Alkorta & Garbisu, 2001). The salt marsh vegetation is dominated by halophytes that have the capacity to tolerate high salt concentration (Flowers & Colmer, 2008) and their potential as bioindicators/biomonitors of contamination (Anjum *et al.*, 2013) or in the clean-up of petroleum hydrocarbon contamination have been studied (Oliveira & Mendelssohn, 2009, Al-Mailem *et al.*, 2010, Couto *et al.*, 2011, Ribeiro *et al.*, 2013). Plants exudates (e.g, sugars, organic acids and vitamins) enhance microbial activity promoting hydrocarbon reduction in contaminant areas. Therefore, one of the mechanisms responsible for phytoremedition is the increase of hydrocarbon degrading bacteria populations containing catabolic genes responsible for hydrocarbon degradation (Siciliano *et al.*, 2002).

The stimulation of indigenous microorganisms capable of degrading pollutants or the addition of engineered microorganisms to enhance biodegradation has been reported (Barac *et al.*, 2004, Germaine *et al.*, 2009, Afzal *et al.*, 2013). *Pseudomonas* species have been shown to be capable to degrade a range of organic pollutants, including PAH,

halogenated derivates and recalcitrant organic residues (Bhattacharya *et al.*, 2003), and were frequently reported in PAH-impacted environments (Ma *et al.*, 2006, Coelho *et al.*, 2011, Dubinsky *et al.*, 2013, Oliveira *et al.*, 2014).

Halimione portulacoides (L) Allen, a low perennial shrub of the Chenopodiaceae family, highly abundant in salt marshes along the Atlantic coast of Europe (Waisel, 2012), is a promising species for the application of phytoremediation in salt marsh sediments. *H. portulacoides* has been the subject of a variety of studies due to its potential to accumulate several metals (Cacador *et al.*, 2009, Valega *et al.*, 2009, Anjum *et al.*, 2011), and is also a good candidate to use in phytoremediation of hydrocarbon contamination areas. In this study, 2-methylnaphthalene was used as model of hydrocarbon sediment contamination. Naphthalene is the simplest and most widespread environmental pollutant member of the chemical family of polycyclic aromatic hydrocarbons (PAHs) and its methyl-substituted derivate is among the most toxic components in crude and fuel oils (Mahajan *et al.*, 1994).

The objective of this study was to test, under controlled microcosms conditions, if the inoculation of *H. portulacoides* with a hydrocarbon-degrading bacterium would mitigate the negative effects of the exposure of plants to hydrocarbon contamination.

Material and Methods

Samples of *Halimione portulacoides* were collected from an estuarine system (Ria de Aveiro, Portugal, 40º36'N, 08º45'W) during late autumn. In order to obtain grafts of *H. portulacoides*, stems with leaves were cut in small pieces (aprox. 6-8 cm) that were placed in bottles with ¼ Hoagland nutrient solution with the following composition: 1.5 mM KNO_3, 1 mM Ca $(NO_3)_2$, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4.7H_2O$, 50 mM KCl, 25 mM H_3BO_3, 2 mM $MnSO_4$. H_2O, 2 mM $ZnSO_4.7H_2O$, 0.5 mM $CuSO_4.5H_2O$, 0.5 mM $(NH_4)_6$ $Mo_7O_{24}.4H_2O$ and 20 mM FeNaEDTA (Duarte *et al.*, 2007). The plants were kept at room temperature under natural light for approximately five months in order to allow the development of new root biomass. Test-sediment was collected from the same site sieved through 2.00 mm. The sediment had a moisture capacity of 22.47%, organic matter content of 4.25% and a pH 7.12. The sediment texture corresponds to mud with 58% of fines (Oliveira *et al.*, 2014).

The experimental design was randomized, with grafts divided in 4 treatments: control (Ctl – sediment with plants); sediment with inoculated plant (I); sediment contaminated with 2-methylnaphthalene and with plant (MtN); sediment contaminated with 2-methylnaphthalene and with inoculated plant (MtN_I). The experiment was conducted for eight weeks.

Treatments

In order to simulate PAH contamination, sediments (MtN and MtN_I) were spiked with 2-methylnaphthalene (final concentration 10 $\mu g\ g^{-1}$). To achieve a uniform spiking, 2-methylnaphthalene was dissolved in acetone and added to 1/4 of total sediment. The acetone was allowed to evaporate overnight. The contaminated sediment (1/4) was well mixed with additional sediment (3/4) (Brannock, 2004). Each container took 150 g of sediment.

Pseudomonas sp. SN31 strain was obtained after enrichment with 10 mg of 2-methylnaphthalene crystals as the sole carbon source as described in a previous study (Oliveira *et al.*, 2014). The 16S rDNA ribosomal sequence used to identify the isolate is available at the GenBank under the accession number: EF628000. Before planting, the plant grafts were divided in two sets. In one, plants were treated with freshly grown *Pseudomonas* sp. SN31 cells (10^7) by dipping the roots in a 0.85% NaCl solution, over 72 h. In other set the roots were dipped only in sterile NaCl solution (Fig. 4.S1).

Finally, plants were transferred to PAH-spiked or control sediment and maintained under natural light and room temperature. During the course of the experiment, two days per week all treatments were subjected to irrigation with Hoagland´s solution (Fig. 4.S2).

Quantification of 2-methylnaphthalene

Sediment was analyzed for 2-methylnaphthalene using a Soxhlet extraction followed by a GC-MS analysis. Four sediment samples of each treatment were homogenized and freeze-dried before extraction. Deutorated naphthalene-d_8 surrogate standard was added to sediment samples to monitor the procedures of sample extraction, cleanup and analysis. In a Soxhlet apparatus 5 g dried and homogenized sediment

treatment was extracted with 150 ml dicloromethane. Prior to extraction, the sediment was spiked with 25 µl (2 µg/ml) deuterated recovery surrogate standard (naphthalene-d_8). Activated copper (Schubert *et al.*, 1998) were added to the collection flask to avoid sulfur interferences when using mass chromatography. After extraction, the sample extracts were concentrated by rotary evaporation up to a volume of about 2-3 ml, and change solvent into 10 ml n-hexane which further reduce to approximately 1-2 ml. The concentrated hexane extract with 2-methylnaphthalene was separated using a 2:1 alumina/silica gel column with anhydrous sodium sulfate overlaying the alumina (remove small quantities of water). This column was used to clean-up and fractionate the extract. Elution was performed using 15 ml of hexane to yield the first fraction and followed by 30 ml of dicloromethane/hexane (1:1). The sample volume was reduced to 1 ml by rotary vacuum evaporator and further to 0.2 ml with a gentle purified nitrogen stream. A known quantity (2 mg/ml) of the internal standard, hexamethylbenzene, was added prior to GC-MS. GC-MS analysis was carried using an Agilent Network GC system, namely an Agilent 6890 gas chromatograph equipped with a mass selective detector (MSD 5973) in the selective ion mode (SIM). The 2-methylnaphthalene was separated using a VF-5MS fused silica column (30 m x 0.25 mm i.d and 0.25 µm film thickness). GC/MS operating conditions were as follows: injector and transfer-line temperatures were maintained at 300 ºC. The oven temperature programmed was initially isothermal at 60 ºC for 1 min, increased to 200 ºC at a rate of 10 ºC/min (hold for 2 min), and then kept isothermal at 300 ºC for 3 min with a rate of 20 ºC/min. Helium as the carried gas a flow rate of 1.3 mL min^{-1}. A 1 µL sample was manually injected in the splitless mode with a 1 min solvent delay. The mass spectra of 2-methylnaphthalene was acquired at the electron impact (EI) mode at 70 eV. The mass scanning ranged between m/z 20 and m/z 500. The naphthalene-d_8 surrogate recovery was 90 ± 6 %.

Photosynthetic performance

Chlorophyll a fluorescence was monitored by PAM fluorometry as an indicator of photosynthetic performance, using an imaging chlorophyll fluorometer (Open FluorCAM 800-O/1010; Photon Systems Instruments). The equipment comprises a

computer-operated control unit (SN-FC800-082, PSI), an 2/3" CCD camera (CCD381, PSI) attached to an F1.2 (2.8-6 mm) objective (Eneo) and four 13 x 13 cm LED panels emitting red light (emission peak at 621 nm, 40 nm bandwidth). Two LED panels (MLS13x13-016A/B) provided the modulated measuring light (< 0.1 µmol m^{-2} s^{-1}) and the other two (SL3500-179/180) provided saturating pulses (> 7500 µmol m^{-2} s^{-1}, 0.6 s). Chlorophyll a fluorescence was filtered using a filter system (composed by a high pass filter (695 nm) and a low pass filter (780 nm) and images (12 bit, 512 x 512 pixels) were captured and processed using FluorCam7 software (Photon Systems Instruments). Experimental procedure was carried out under the control of user-defined automated measuring sequences (protocols) with the ability to define measurement intervals and irradiance (Nedbal *et al.*, 2000). For each treatment, five replicate of *H. portulacoides* leaves under the same experimental conditions were dark adapted for 5 minutes, before the maximum quantum yield of photosystem II (PSII) could be measured (F_v/F_m). A user defined protocol, with a total duration of 2s, was used to estimate F_v/F_m and allowed minimum fluorescence (F_o), to be measured for 1,4 seconds and then a 600 ms light saturating pulse (SP) was applied, which allowed the determination of maximum fluorescence (F_m) values. F_v/F_m was calculated following Kitajima & Butler (1975). After the estimation of F_v/F_m, rapid light-response curves (RLCs) were performed immediately on the same samples. RLCs were built using 14 increasing actinic light steps, from 53 to 1531 µmol m^{-2} s^{-1}, with each light step taking 10 sec, after which an SP was applied, allowing the estimation of steady state fluorescence (F_s) and maximum light-adapted fluorescence (F_m') for each light step. The relative electron transport rate ($_r$ETR) was calculated for each light step, as $_r$ETR = $[(F_m' - F_s)/F_m]'$*PAR, with $(F_m' - F_s)/F_m$, being the PSII effective quantum yield (Genty *et al.*, 1990). Light curve parameters, such as α (initial slope), $_r$ETR$_m$ (maximum $_r$ETR) and E_k (light-saturation parameter) where quantified by fitting the model of Eilers & Peeters (1988), using the MS Excel Solver.

Total community DNA (TC-DNA) extraction

Four samples of 0.5 g of rhizosphere sediments were subject to TC-DNA extraction using a MoBio Ultraclean™ soil DNA kit (Cambio) following the manufacturer´s

instruction. The endophytic community was recovered from fresh roots. Root material was vigorously washed in distilled water (5 min) and root surface was disinfected by sequential washing with 95% ethanol and 1% sodium hypochlorite supplemented with one droplet Tween 80, and rinsed three times in sterile distilled water (Barac *et al.*, 2004). A 100 µL sample of final rinsing water was spread-plated on 869 medium (Mergeay *et al.*, 1985) to check the efficiency of disinfection. The endophytic TC-DNA extraction was performed using a bead-beating protocol previously described in (Phillips *et al.*, 2006). About 2 g of surface disinfected root material was macerated in liquid nitrogen. The cell lysis was achieved by a combination of bead-beating, proteinase K (20 mg mL^{-1}) and sodium dodecyl sulfate (20%). Procedures cellular debris and proteins were precipitated with aqueous 7.5 M ammonium acetate. DNA was subsequently precipitated with isopropanol, washed with 70% cold ethanol and re-suspended in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The endophytic TC-DNA was purified with the GeneClean Spin Kit (MP Biomedicals).

PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE)

A nested PCR approach was used to amplify the 16S rRNA gene sequence from rhizosphere sediments and endophytic communities (Gomes *et al.*, 2008). Briefly, in the first PCR the universal bacterial primers U27 and 1492R were used. The amplicons obtained was used as template for a second PCR with the bacterial DGGE primers 984F-GC and 1378R (Heuer *et al.*, 1997). PCR proceeding and amplification conditions were described in (Oliveira *et al.*, 2014).

The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel containing 6–10% acrylamide with a gradient of 40–58% of denaturants. The run was performed in Tris-acetate-EDTA buffer at 60 °C at a constant voltage of 80 V for 16 h using the DCode System (Universal Mutation Detection System, Bio-Rad). The DGGE gels were silver stained (Heuer *et al.*, 2001). The processing of the scanned DGGE gels was carried out using the Bionumerics software 6.6 (Applied Maths).

Detection of genes encoding for PAH degradation

The presence of genes encoding the large subunit of naphthalene-1,2-dioxygenase (*nahAc*) and alpha subunit of the PAH ring-hydroxylating dioxygenases genes, both related to PAH degradation, was detected by PCR using the primers and reaction conditions described by Wilson *et al*. (1999) and Cébron *et al*. (2008), respectively and summarized in Oliveira *et al.*, (2014).

Statistical analysis

After analysis of DGGE gels the data matrix of band abundance (band positions and their corresponding intensities) per sample was log10 (x + 1) transformed and a distance matrix was constructed using the Bray-Curtis index with the vegdist() function in the vegan package (Oksanen *et al.*, 2011) in R version 3.0.1 (http://www.r-project.org/; checked 2013/09/13). Variation in bacterial composition as response to different treatments in rhizosphere sediments and root endophytic communities was visually assessed with Principal Coordinates Analysis (PCO) using the cmdscale() function in R using the Bray-Curtis distance matrix as input. Significant differences between treatments in the communities of different microhabitats were tested using the adonis() function in vegan. The adonis() function performs an analysis of variance with distance matrices using permutations that partition the distance matrices among sources of variation. In the adonis() analysis, the Bray-Curtis distance matrix of band composition was the response variable with type of treatments the independent variable. The number of permutations was set at 999; all other arguments used the default values set in the function. Plant fluorescence parameters and 2-methylnaphthalene concentration data was included in an analysis of variance (ANOVA) model to determine the specific effects of each treatment. ANOVA analysis were performed using aov() function of the R.

Results

Growth, maximum quantum yield of PSII and light curve parameters

Halimione portulacoides grew well in both controls (non-contaminated) and 2-methylnaphthalene contaminated sediments. At the end of experiment plants appeared

healthy, with development of news leave and shoots (Fig. 4.S2). Fluorescence parameters were used to determine if 2-methylnaphthalene had a significant negative impact on plant health and photophysiology (Table 4.1). Maximum quantum yield of photosystem II (F_v/F_m) was evenly high in all treatments with an average of 0.788, without significant differences between treatments. The $_rETR_m$ parameter used as a descriptor of the maximum photosynthetic rate, decrease in all treatments from the beginning to the end of experience (Table 4.1).

Table 4.1 Chlorophyll a fluorescence parameters initial and after eight weeks of 2-methylnaphthalene contamination. Maximum quantum yield of PSII (F_v/F_m) and light curve parameters (α, initial slope), $_rETR_m$ (maximum relative electron transport rate) and E_k, light-saturation) for *H. portulacoides*.

	Initial (T0)	Treatments (after eight weeks)				
	P	P_I	Ctl	I	MtN	MtN_I
F_v/F_m	0.78 ± 0.03	0.77 ± 0.03	0.77 ± 0.02	0.82 ± 0.01	0.78 ± 0.01	0.82 ± 0.01
α	0.59 ± 0.10	0.50 ± 0.05	0.47 ± 0.15	0.50 ± 0.16	0.30 ± 0.10	0.46 ± 0.17
$_rETR_m$	107.75 ± 6.28	125.21 ± 15.53	72.84 ± 16.52	45.75 ± 21.03	77.27 ± 12.36	58.39 ± 6.55
E_k	170.29 ± 18.69	222.79 ± 56.45	144.84 ± 15.10	82.07± 36.34	278.26 ± 99.99	124.44 ± 31.94

P, plants before planting; P_I, plants before planting with isolate; Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant

Concentration of 2-methylnaphthalene

The final 2-methylnaphthalene concentrations in spiked sediments were lower ($p < 0.001$) than at the beginning of the experiment (Table 4.2). The initial concentration of 2-methylnaphthalene in sediment was approximately 48 ng g dwt^{-1} and decrease to approximately 12 ng g dwt^{-1} at the end of experiment (Table 4.2) in both amendmdent sediments (MtN and MtN_I). In the treatments without added 2-methylnaphthalene, the concentrations at the beginning and at the end of the experiment were not significantly different (Table 4.2).

Table 4.2 Quantification of 2-methylnaphthalene (ng g dwt^{-1}).

	Initial		Treatments (after eight weeks)			
	Sed	Sed_MtN	Ctl	I	MtN	MtN_I
2-methylnaphthalene						
	2.80 ± 0.80	47.99 ± 6.27	3.38 ± 0.24	2.39 ± 0.90	11.74 ± 3.45	11.72 ± 4.28

Sed, sediment before treatments (negative control); Sed_MtN, sediment contaminated with 2-methylnaphthalene before treatments; Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant

DGGE analysis of the bacterial community

The effect of 2-methylnaphthalene on the bacterial community structure was determined using 16S rRNA gene-based PCR-DGGE for rhizosphere sediments and endophytic community at the end of the experiment. In order to determine differences between the bacterial community fingerprints, PCO was performed based on intensity of each DGGE band under the different conditions and microhabitats. PCO analysis shows that depending on the microhabitat, treatments have distinctly influenced bacterial community responses. The rhizosphere communities (Fig. 4.1) were generally conserved and a detectable shift in community structure only occurred under the combined pressure of 2-methylnaphthalene amendment and bacterium inoculation (Adonis $F_{3,12}$ =3.60, $P < 0.001$, $R_2 = 0.607$). The PCO analysis of the endophytic community (Fig. 4.2) showed a clear separation of one cluster corresponding to the controls (Ctl) and another cluster including all other treatments (Adonis $F_{3,12}$ =3.60, $P < 0.001$, $R_2 = 0.444$) with inoculum and 2-methylnaphthalene separately or both additions at the same time.

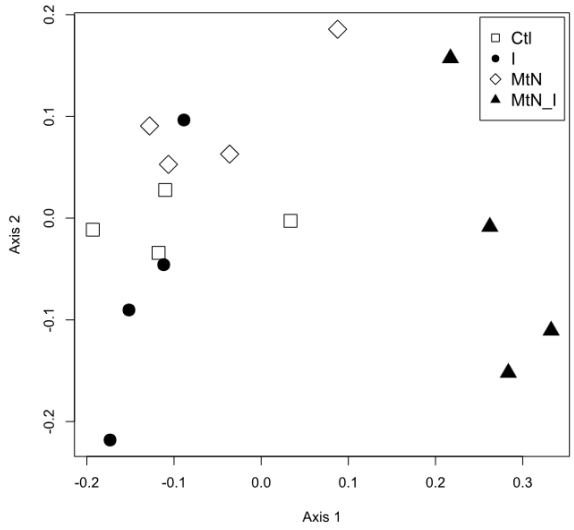

Fig. 4.1 Ordination based on principal coordinate analysis (PCO) showing the comparison between DGGE fingerprints from rhizosphere community in the four treatments (Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant).

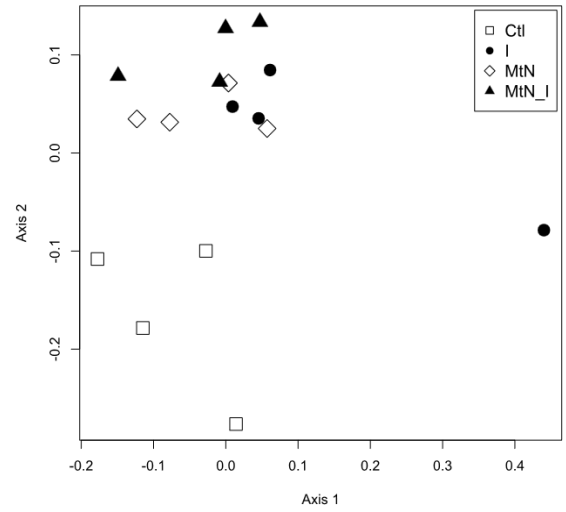

Fig. 4.2 Ordination based on principal coordinate analysis (PCO) showing the comparison between DGGE fingerprints from endophytic community in the four treatments (Ctl, sediment with plant; I, sediment with inoculated plant; MtN, sediment contaminated with 2-methylnaphthalene and with plant; MtN_I, sediment contaminated with 2-methylnaphthalene and with inoculated plant).

Detection of genes encoding for PAH degradation

The results of the detection of genes encoding for PAH degradation are summarized in Table 4.3. The only gene detected was that corresponding to the large subunit of naphthalene-1,2-dioxygenase (*nahAc*) and positive results were only obtained for treatments corresponding to 2-methylnaphthalene (MtN-I) amendment.

Table 4.3 Detection of genes encoding PAH degradation enzymes in each treatment.

	Rhizosphere				Endophytic			
Treatments (after eight weeks)	Ctl	I	MtN	MtN_I	Ctl	I	MtN	MtN_I
nahAc	-	-	++++	++++	-	-	-	++++
PAH-RDHα-GN	-	-	-	-	-	-	-	-
PAH-RDHα-GP	-	-	-	-	-	-	-	-

-, no detection in all replicates; +++, detection in three replicates; ++++, detection in all replicates (n = 4)

Discussion

Salt marsh sediments represent a phytoremediation challenge due to their particular biological, physical and chemical specificities. These habitats are constantly subjected to very different environmental pressures such as flooding, salinity fluctuations and contamination with organic and inorganic compounds. Considering that hydrocarbon pollution has severe effects on salt marsh vegetation, the main goal of this study was to determine whether the inoculation of a salt marsh halophyte with a hydrocarbon-degrading bacterial strain could improve its overall degradation potential without affective its health status.

Plant condition

Several studies have reported significant reduction of the growth of salt marsh plants subjected to high petroleum hydrocarbons dosage (Mendelssohn *et al.*, 1990, Lin & Mendelssohn, 1996, Pezeshki *et al.*, 2000, Lin & Mendelssohn, 2009). Nevertheless, the type, quantity and time of exposure of the hydrocarbon contamination may be determinant (Lin & Mendelssohn, 1998). The tolerance limits of the salt marsh halophyte *Juncus roemerianus* is estimated to be between 160 and 320 mg diesel g^{-1} dry sediment. Above this limit, growth can be significantly affected (Lin & Mendelssohn,

2009). Although the susceptibility of *Halimione portulacoides* is not well characterized, plants were not significantly affected by the concentration of 10 µg g^{-1} of 2-methylnaphthalene used in this work, as revealed by the F_v/F_m descriptor of photosynthetic efficiency. This means that quantum efficiency of the light reactions of photosynthesis on the contaminated plants was not affected. Information on the photosynthetic potential at different light regimes can also be inferred from the light curve parameters, especially the $_rETR_m$. The results of this parameter indicate that there was some variation of the photosynthetic potential during the course of the microcosm experiments. This can be explained as an acclimation phenomenon.

In this study, the concentration of added PAH was intentionally low in order to represent realistic levels of contamination in moderately polluted estuaries. The results indicate this PAH load was generally well tolerated by the plants. A study of the effect of oil contamination (range 0.3 to 2.73 L m^{-2}) on the photosynthetic activity of *Salicornia virginica* produced similar results (Rosso *et al.*, 2005) indicating that plants can tolerate moderate concentrations of hydrocarbon without significant impairment of the photosynthetic function. In a field trial, there was only a modest variation on the photosynthetic rate in a group of plants subjected to petroleum contamination and inoculated with PGPR (plant growth promoting rhizobacteria) (Gurska *et al.*, 2009). A different result was obtained in a study in which the effects of oil on *Spartina alterniflora* photosynthesis were measured. In this case, despite a full year of recovery, plants exhibited chronic stress which depressed photosynthesis (Biber *et al.*, 2012).

Hydrocarbon clearance

The comparison of the concentration of 2-methylnaphthalene at the beginning and at the end of the experiments shows that, in the presence of the plant, there was a 75% reduction after 8 weeks in microcosm experimentally amended with 2-methylnaphthalene (MtN and MtN-I). Several studies have demonstrated that phytoremediation is an efficient strategy for the removal of hydrocarbons from contaminated sediments (Taghavi *et al.*, 2005, Lin & Mendelssohn, 2009) in comparison to other bioremediation approaches (Germaine *et al.*, 2009, Moreira *et al.*, 2011). The increase of root biomass produces considerable amounts of exudates and

consequently stimulates bacterial activity in the rhizosphere microhabitat, which might contribute to enhanced OH capacity (Escalante-Espinosa *et al.*, 2005, Moreira *et al.*, 2011). On the other hand, a study using the same plant demonstrated that *H. portulacoides* can interfere with the degradation of higher molecular weight PAHs by sediment microorganisms and this was interpreted as an indication of competition for nutrients between plants and microorganisms (Mucha *et al.*, 2011).

The fact that inoculation did not enhance 2-methylnaphthalene degradation by the plant can be related with the inoculation method or environmental factors. Other studies report that the failure of bacteria inoculation in contaminated sites can be a matter of inoculum size (Mishra *et al.*, 2001) or strong influence of soil characteristics, such as the soil type, particle sizes or organic matter content (Afzal *et al.*, 2012, Afzal *et al.*, 2013). May be the inoculant would have been more helpful if 2-methylnaphthalene concentrations were very high.

Structure of bacterial communities

In this experiment the contamination had a differentiated impact on the structure of bacterial communities, depending on the microhabitat (endophytic or rhizosphere sediments). The presence of contaminants is known to modify the composition and activity of sediment bacterial communities (Gomes *et al.*, 2005). Moreover, plants may enhance pollutant degradation by positively affecting the abundance, diversity and activity of specific microorganism in the surrounding rhizosphere (Siciliano *et al.*, 2002, Mucha *et al.*, 2011) and within plants roots (Phillips *et al.*, 2008, Ryan *et al.*, 2008). The results of this work indicate that endophytic and rhizosphere communities exhibited different responses to inoculation and/or to 2-methylnaphthalene amendment. The rhizosphere microbiota was more resilient and changes only occurred when two sources of variability (contamination and inoculation) were imposed. The inoculant used in this experiment was actually isolated from the endosphere of *Sarcocornia perennis* subsp. *perennis* root in a hydrocarbon-impacted salt marsh of Ria de Aveiro (Oliveira *et al.*, 2014). Therefore, it is expectable that this strain might also be present in the roots of the plants used in this study and that the inoculation alone did not represent a significant shift in community structure. Also, the rhizosphere communities are naturally enriched

in hydrocarbon-degrading bacteria (Daane *et al.*, 2001, Ma *et al.*, 2010, Ribeiro *et al.*, 2011) and the amendment with realistic concentrations of 2-methylnaphthalene may not represent a significant challenge to the rhizosphere bacterial community. However, when the contaminant and the contaminant-inoculum were combined, there was detectable change in the bacterial community that could indicate a stress-relieve response.

The PCO analysis of endophytic communities shows that the community reacted to contamination and to inoculation and also to the combination of both manipulations. These results suggest that endophytic communities are more sensitive to selective pressures applied separately that can correspond to a successful competition between inoculated bacteria and indigenous endophytic bacteria for plant roots. The endophytic communities are unique to each plant species and differences in their distribution and diversity can be due to plant-specific factors, metabolic pathways, nutrient availability and composition of root exudates (Günther *et al.*, 1996, Rosenblueth & Martínez-Romero, 2006). The amendment of an aromatic hydrocarbon causes a change in the composition of the endophytic community that may correspond to a selective response of particular hydrocarbon degrading strains. The relations between plant-specific factors, composition of the endophytic bacterial community and potential for hydrocarbon degradation have already been demonstrated in prairie plants (Phillips *et al.*, 2008). The inoculum used in the present work is a hydrocarbon endophytic bacterium (Oliveira *et al.*, 2014). Therefore, the observed change in the endophytic community in inoculated treatments suggests that the internalization of inoculant cells did occur during the experiment, even without experimental amendment with 2-methylnaphthalene, and that the inoculant may be a promising root colonizer.

Hydrocarbon degradation genes

Naphthalene dioxygenase genes, as representatives of the family of catabolic genes involved in hydrocarbon degradation, were analyzed to assess the hydrocarbon-degradation potential of rhizosphere and endophytic bacterial communities. At the end of the 8-week experiment, naphthalene dioxygenase genes were only detected in 2-methylnaphthalene treatments, and in the case of the endophytic community, in

inoculated plants. The positive detection only in contaminated sediments can be due to the increase of naphthalene degradation genes number and the enhancement of horizontal gene transfer in response of 2-methylnaphthalene contamination. In fact, it has been reported that biodegradation potential is enhanced by horizontal gene transfer and development of an efficient degrading microbial community upon contamination (Top *et al.*, 2002, Top & Springael, 2003). The number of *nahAc* gene copies is directly related with the rate of naphthalene degradation (Park & Crowley, 2006) and the response to 2-methylnaphthalene in terms of *nahAc* gene copy number may be interpreted as an adaptative stress response. The fact that naphthalene degradation genes in endophytic communities were only detected in the treatment corresponding to inoculated plants in spiked sediments can be related with selection of naphthalene endophytic degraders in response to contamination.

Conclusions

In conclusion, the salt mash plant *H. portulacoides* can influence microbial communities by favoring the development of hydrocarbon-degrading bacteria which may contribute to hydrocarbon clearance in salt marsh sediments. The hypothesis that the inoculation of the plants with the PAH-degrading endophytic bacterium would enhance 2-methylnaphthalene degradation could not be demonstrated. However, the inoculation did have an impact on the structural diversity of endophytic communities. This result indicates that this strain is a good plant colonizer. Because it carries genetic determinants for hydrocarbon degradation (e.g. plasmids) that can be horizontally transferred within the community, it may represent a promising candidate for microbe-assisted phytoremediation approaches.

Acknowledgements

This work had financial support from CESAM research unit (PEst-C/MAR/LA0017/2011), QOPNA research unit (project PEst-C/QUI/UI0062/2011), Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-CLI/107916/2008 (http://alfa.fct.mctes.pt) and COMPETES-(FCOMP-01-0124-

FEDER-008657). Financial support to V. Oliveira (SFRH/BD/46977/ 2008) was provided by FCT in the form of a PhD grant.

CHAPTER 4

Evaluation of plant-microbe interactions in 2-methylnaphthalene contaminated sediments

Supplementary Material

Fig. 4.S1 *Halimione portulacoides* inoculated with *Pseudomonas* sp. endophytic degrader.

Fig. 4.S2 Plant/sediment microcosm at the end of the experiment.

Chapter 5

CHAPTER 5
Conclusions

The deposition and accumulation of contaminants, such as hydrocarbons, in salt marsh sediments is a recurrent global concern issue. Therefore, the relations between plants and microorganisms involved in hydrocarbon removal or degradation have for long been followed with interest. The rhizosphere effect in the process of OH (oil hydrocarbon) degradation has been progressively recognized. However, only very few studies have focused on the rhizosphere of salt marsh vegetation (Daane *et al.*, 2001, Mucha *et al.*, 2011, Ribeiro *et al.*, 2011) and practically none on the associated endophytic bacterial communities. Phytoremediation is an inexpensive and "green" approach based on the interactions between plants, their associated indigenous degrading microorganisms (rhizo- and endosphere) and pollutants. The potential of salt marsh halophytes to clean-up petroleum hydrocarbons contamination have been studied (Oliveira & Mendelssohn, 2009, Al-Mailem *et al.*, 2010, Couto *et al.*, 2011, Ribeiro *et al.*, 2013). Moreover, the use of endophytic-derived inoculants to reduce the pollutant toxicity to plants and increase biodegradation has been demonstrated (Barac *et al.*, 2004, Taghavi *et al.*, 2005).

The main goal of this study is the characterization of relevant plant-microbe interactions in oil-impacted salt marshes and the understanding of the effect of rhizosphere and endosphere bacteria in the role of salt marsh plants as potential phytoremediation agents. In order to achieve the objectives, the methodological approach combined field observations with experimental manipulations, conducted in plant sediment microcosms.

Field work

In the field, this study addressed some general questions: How do plant species- and OH pollution affect indigenous sediment bacterial composition and degradation potential in sampling sites exposed to different levels of contamination? How do endophytic communities respond? Which genetic factors are involved in endophytic OH degradation capacity?

The shallow estuary-coastal lagoon Ria de Aveiro is composed of four main channels that divide into an intricate network of narrow and shallow secondary cannels separated by mud banks and salt marshes. The Aveiro long-distance port facilities are installed at the outer section of the estuary and elevated hydrocarbon contamination has been reported at this area (Pacheco & Santos, 2001). Also, other antropogenic inputs, such as recreational navigation, urban runoff and intense commercial shipping activity, cause a continuous efflux of OH.

The location of the four collecting points was chosen to reflect different levels of OH pollution among the areas where the two studied halophytes could be found. The halophytes *Halimione portulacoides* (L.) Allen, and *Sarcocornia perennis* subsp. *perennis* (P. Mill) are widely represented in temperate estuaries and prevalent species in Ria de Aveiro salt marshes (Silva *et al.*, 2009). Both Chenopodiaceae, these species can tolerate high salinity and water potentials (Rubio-Casal *et al.*, 2003). By playing a relevant role in organic matter recycling and on nutrient and metal sequestration, they have been proposed for phytoremediation approaches.

Within the field approach, culture-dependent and -independent methods were used to investigate the effects of OH contamination on bacterial communities in salt marsh microhabitats (bulk sediment, rhizosphere and endosphere). The structure and composition of sediment bacterial communities appear to be affected by site related factors (sediment properties and oil hydrocarbon contamination) and plant related factors (exudates composition). Sediment properties (organic content and gran size) influence the bioavailability of hydrocarbons (Kim *et al.*, 1999, Wang *et al.*, 2001). Moreover, plants can influence the hydrocarbon retention around roots (Ribeiro *et al.*, 2011) and affect the composition of the microbial community by selecting the development of hydrocarbon-degrading microbial population in its rhizosphere. The composition of the root exudates released by each plant can underlie the differences of diversity and structure of rhizosphere and endosphere communities of study plants. In a previous work directed to volatile compounds emissions which compose a relevant fraction of root exudates (Steeghs *et al.*, 2004), the high complexity of the chemical composition of root-derived material associated to salt marsh plants (*Spartina maritima* and *Halimione portulacoides*) was demonstrated, indicating that the quality and quantity of root derived organic matter may be a relevant driver of bacterial dynamics in salt

marsh sediments. Phillips et al., (2012), suggested that the patterns of exudation released by *Elymus angustus* (wildrye) and *Medicago sativa* (alfalfa) are the driving factors behind the degradation traits of bacteria.

In terms of degradation potential, the *in silico* metagenome analysis of 16 sRNA gene amplicons obtained in this study showed enrichment of genes involved in the process of PAH degradation in the rhizosphere of halophyte plants. The endophytic community showed potential degradation capacity with the presence and abundance of genes encoding for PAH-degrading enzymes which were plant species/contaminant dependent.

Enrichment cultures of endophytic bacteria were prepared in order to obtain OH-degrading bacterial strains with potential for later use in the microcosm experiments. The endophytic strains retrieved revealed a predominance of *Pseudomonas* sp. *Pseudomonas* is frequently isolated from oil-contaminated sediments (Ma *et al.*, 2006, Gomes *et al.*, 2007), even in other microhabitats oil contaminated of Ria the Aveiro (Coelho *et al.*, 2011, Domingues *et al.*, 2013). Same endophytic strains revealed degradation potential with an importance of *nah* genotypes in the PAH degradation in salt marsh plants. A dominance of *nah*-like genotypes in isolates after enrichment with naphthalene has been reported (Ma *et al.*, 2006, Ni Chadhain *et al.*, 2006). Plasmid-mediated mineralization of aromatic hydrocarbon (AH) is a major process of oil hydrocarbon biodegradation. So, the horizontal gene transfer mediated by catabolic plasmid plays an important role in AH degradation capacity of salt marsh bacterial communities. The detection, in same endophytic isolates, of IncP-9 catabolic plasmids, a plasmid family known for carrying genes encoding for enzymes involved in hydrocarbon degradation, suggests that horizontal gene transfer can drive the spread of *nah* genes in these habitats and probably contribute to the process of bacterial endophytic adaptation and *in planta* OH decontamination.

Based on the results obtained from the field, the following conclusions can be drawn:

- The structure and composition of microbial communities in salt marsh microhabitats (bulk sediment, rhizosphere and endosphere) are affected by site-related and plant-related factors.

- Halophyte colonization could be an important driver of the structure of hydrocarbonoclastic bacterial community in salt marsh sediments, which can be exploited for *in situ* phytoremediation of OH in salt marsh environments.
- The culturable fraction of endophytic PAH degraders revealed the presence of genetic determinants for hydrocarbon degradation (plasmids and *nah* genes), suggesting a potential ecological role of bacterial-plant symbionts on the process of colonization and in plant survival in urban estuarine areas exposed to OH contamination.

Microcosm component

A plant-sediment microcosm experiment was designed in order to experimentally manipulate the concentration of a representative aromatic hydrocarbon (2-methylnaphthalene) and test if the introduction of an indigenous endophytic hydrocarbon-degrading bacterium can reduce contaminant toxicity to plants and enhance contaminant degradation.

Plant condition was not significantly affected by the addition of a moderate concentration of 2-methylnaphthalene, leading to the conclusion that *Halimione portulacoides* plants are tolerant to a concentration of 10 µg g^{-1}. At the end of the experiment, the concentration of 2-methylnaphthalene in spiked sediments was reduced by approximately 75 %. Phytoremediation studies suggest that plants have the ability to stimulate PAH-degrading microorganisms, ultimately enhancing hydrocarbon bioremediation (Phillips *et al.*, 2006, Ribeiro *et al.*, 2011). In fact, at the end of the experiment, the detection of catabolic genotypes in bacteria from plants corresponding to particular microcosm treatments (spiking with 2-methylnaphthalene) suggests that hydrocarbonoclastic populations were enriched in response to contamination.

The use of a microbial inoculant or a consortium, in interaction with plants, to promote degradation of hydrocarbon contaminants has been well explored, with a variety of results reported in the literature. A genetically modified endophytic strain of *Burkholderia cepacia* was shown to increase the tolerance of *Lupinus luteus* to toluene, while decreasing toluene evapotranspiration (Barac *et al.*, 2004). Another study concluded that despite the failure of endophytic inocula to survive in detectable numbers, transference of their degradative plasmids to endogenous endophytic

communities occurred (Taghavi *et al.*, 2005). So, the efficient colonization of plants by microbial pollutant-degraders can represent a major contribution to plant survival and hydrocarbon degradation. The viability and colonization efficiency of an applied strain, however, depends on a variety of factors: plant and bacterial genotypes, inoculation mode and the physicochemical properties of the soil environment. The inoculation method and soil type can exert a major effect on plant growth, bacterial survival and activity and hydrocarbon degradation. The importance of inoculation procedures and soil type should be considered in the design of efficient phytoremediation applications (Afzal *et al.*, 2011, Afzal *et al.*, 2013).

Based on the results obtained in the microcosm approach the following conclusions can be drawn:

- *H. portulacoides* can influence the structure of microbial communities by selectively enhancing the development of hydrocarbons degrading populations that can contribute to hydrocarbon degradation and removal in salt marshes.
- The enhancement of 2-methylnaphthalene degradation by inoculation of the plants with the PAH-degrading endophytic bacteria could not be demonstrated.
- An efficient colonization of the hydrocarbon-degrading *Pseudomonas* spp. strain can be inferred from the changes imposed on the structure of the indigenous endophytic communities, at the end of the microcosm experiment, indicating that this strain may be regarded as good colonizer of *H. portulacoides* roots.

REFERENCES

Acosta-González A, Rosselló-Móra R & Marqués S (2013) Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the Prestige oil spill. *Environmental Microbiology* **15**: 77-92.

Aeckersberg F, Bak F & Widdel F (1991) Anaerobic oxidation of saturated hydrocarbons to CO2 by a new type of sulfate-reducing bacterium. *Archives of Microbiology* **156**: 5-14.

Afzal M, Yousaf S, Reichenauer TG, Kuffner M & Sessitsch A (2011) Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. *Journal of Hazardous Materials* **186**: 1568-1575.

Afzal M, Yousaf S, Reichenauer TG & Sessitsch A (2012) The inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel oil. *International Journal of Phytoremediation* **14**: 35-47.

Afzal M, Khan S, Iqbal S, Mirza MS & Khan QM (2013) Inoculation method affects colonization and activity of *Burkholderia phytofirmans* PsJN during phytoremediation of diesel-contaminated soil. *International Biodeterioration & Biodegradation* **85**: 331-336.

Aislabie J, Saul DJ & Foght JM (2006) Bioremediation of hydrocarbon-contaminated polar soils. *Extremophiles* **10**: 171-179.

Al-Mailem D, Sorkhoh N, Marafie M, Al-Awadhi H, Eliyas M & Radwan S (2010) Oil phytoremediation potential of hypersaline coasts of the Arabian Gulf using rhizosphere technology. *Bioresource Technology* **101**: 5786-5792.

Alkorta I & Garbisu C (2001) Phytoremediation of organic contaminants in soils. *Bioresource Technology* **79**: 273-276.

Alonso-Gutiérrez J, Figueras A, Albaigés J, Jiménez N, Viñas M, Solanas AM & Novoa B (2009) Bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill. *Applied and Environmental Microbiology* **75**: 3407-3418.

Andria V, Reichenauer TG & Sessitsch A (2009) Expression of alkane monooxygenase (*alkB*) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) grown in diesel contaminated soil. *Environmental Pollution* **157**: 3347-3350.

Anjum NA, Ahmad I, Válega M, Pacheco M, Figueira E, Duarte AC & Pereira E (2011) Impact of seasonal fluctuations on the sediment-mercury, its accumulation and partitioning in *Halimione portulacoides* and *Juncus maritimus* collected from Ria de Aveiro coastal lagoon (Portugal). *Water, Air, & Soil Pollution* **222**: 1-15.

Anjum NA, Ahmad I, Válega M, Mohmood I, Gill SS, Tuteja N, Duarte AC & Pereira E (2013) Salt Marsh Halophyte Services to Metal-Metalloid Remediation: Assessment of the Processes and Underlying Mechanisms. *Critical Reviews in Environmental Science and Technology*. DOI:10.1080/10643389.2013.828271

Ashok B, Saxena S & Musarrat J (1995) Isolation and characterization of four polycyclic aromatic hydrocarbon degrading bacteria from soil near an oil refinery. *Letters in Applied Microbiology* **21**: 246-248.

Baek K-H, Yoon B-D, Cho D-H, Kim B-H, Oh H-M & Kim H-S (2009) Monitoring bacterial population dynamics using real-time PCR during the bioremediation of crude-oil-contaminated soil. *Journal of Microbiology and Biotechnology* **19**: 339-345.

Baek S-H, Kim K-H, Yin C-R, Jeon CO, Im W-T, Kim K-K & Lee S-T (2003) Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions. *Current Microbiology* **47**: 462-466.

Bagwell CE, Piceno YM, Ashburne-Lucas A & Lovell CR (1998) Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. *Applied and Environmental Microbiology* **64**: 4276-4282.

Bahl MI, Burmolle M, Meisner A, Hansen LH & Sorensen SJ (2009) All IncP-1 plasmid subgroups, including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* **62**: 134-139.

Bais HP, Weir TL, Perry LG, Gilroy S & Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology* **57**: 233-266.

Bamforth SM & Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology and Biotechnology* **80**: 723-736.

Banks M, Schwab P, Liu B, Kulakow P, Smith J & Kim R (2003) The effect of plants on the degradation and toxicity of petroleum contaminants in soil: a field assessment. In: *Phytoremediation*, pp. 75-96. Springer Berlin Heidelberg.

Baptista I, Santos AL, Cunha Â, Gomes NC & Almeida A (2011) Bacterial biomass production in an estuarine system: high variability of leucine conversion factors and changes in bacterial community structure during incubation. *Aquatic Microbial Ecology* **62**: 299-310.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J & van der Lelie D (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nature biotechnology* **22**: 583-588.

Barbier EB, Hacker SD, Kennedy C, Koch EW, Stier AC & Silliman BR (2011) The value of estuarine and coastal ecosystem services. *Ecological Monographs* **81**: 169-193.

Baumard P, Budzinski H & Garrigues P (1998) Polycyclic aromatic hydrocarbons in sediments and mussels of the western Mediterranean Sea. *Environmental Toxicology and Chemistry* **17**: 765-776.

Baumard P, Budzinski H, Garrigues P, Dizer H & Hansen P (1999) Polycyclic aromatic hydrocarbons in recent sediments and mussels (*Mytilus edulis*) from the Western Baltic Sea: occurrence, bioavailability and seasonal variations. *Marine Environmental Research* **47**: 17-47.

Bayoumi R (2009) Bacterial bioremediation of polycyclic aromatic hydrocarbons in heavy oil contaminated soil. *Journal of Applied Scienes Research* **5**: 197-211.

Beazley MJ, Martinez RJ, Rajan S, Powell J, Piceno YM, Tom LM, Andersen GL, Hazen TC, Van Nostrand JD & Zhou J (2012) Microbial community analysis of a coastal salt marsh affected by the Deepwater Horizon oil spill. *PloS one* **7**: e41305.

Berg G & Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology* **68**: 1-13.

Bertrand J-C, Bianchi M, Mallah MA, Acquaviva M & Mille G (1993) Hydrocarbon biodegradation and hydrocarbonoclastic bacterial communities composition grown in seawater as a function of sodium chloride concentration. *Journal of Experimental Marine Biology and Ecology* **168**: 125-138.

Bhattacharya D, Sarma PM, Krishnan S, Mishra S & Lal B (2003) Evaluation of genetic diversity among *Pseudomonas citronellolis* strains isolated from oily sludge-contaminated sites. *Applied and Environmental Microbiology* **69**: 1435-1441.

Biber PD, Wu W, Peterson MS, Liu Z & Pham LT (2012) Oil contamination in Mississippi salt marsh habitats and the impacts to *Spartina alterniflora* photosynthesis.

Bimboim H & Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**: 1513-1523.

Bowen JL, Crump BC, Deegan LA & Hobbie JE (2009) Salt marsh sediment bacteria: their distribution and response to external nutrient inputs. *The ISME Journal* **3**: 924-934.

Bowen JL, Morrison HG, Hobbie JE & Sogin ML (2012) Salt marsh sediment diversity: a test of the variability of the rare biosphere among environmental replicates. *The ISME Journal* **6**: 2014-2023.

Brannock JM (2004) Charaterization of root endophytic bacteria. Doctoral Thesis, University of Cincinnati, pp. 87.

Brakstad O & Lødeng A (2005) Microbial diversity during biodegradation of crude oil in seawater from the North Sea. *Microbial Ecology* **49**: 94-103.

Brofft JE, McArthur JV & Shimkets LJ (2002) Recovery of novel bacterial diversity from a forested wetland impacted by reject coal. *Environmental Microbiology* **4**: 764-769.

Budzinski H, Jones I, Bellocq J, Pierard C & Garrigues P (1997) Evaluation of sediment contamination by polycyclic aromatic hydrocarbons in the Gironde estuary. *Marine Chemistry* **58**: 85-97.

Caçador I, Caetano M, Duarte B & Vale C (2009) Stock and losses of trace metals from salt marsh plants. *Marine Environmental Research* **67**: 75-82.

Castle DM, Montgomery MT & Kirchman DL (2006) Effects of naphthalene on microbial community composition in the Delaware estuary. *FEMS Microbiology ecology* **56**: 55-63.

Carman KR, Means JC & Pomarico SC (1996) Response of sedimentary bacteria in a Louisiana salt marsh to contamination by diesel fuel. *Aquatic Microbial Ecology* **10**: 231-241

Cébron A, Norini MP, Beguiristain T & Leyval C (2008) Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHD$_\alpha$) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *Journal of Microbiological Methods* **73**: 148-159.

Cébron A, Beguiristain T, Faure P, Norini MP, Masfaraud JF & Leyval C (2009) Influence of vegetation on the in situ bacterial community and polycyclic aromatic hydrocarbon (PAH) degraders in aged PAH-contaminated or thermal-desorption-treated soil. *Applied and Environmental Microbiology* **75**: 6322-6330.

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**: 351-368.

Chang Y-J, Stephen JR, Richter AP, Venosa AD, Brüggemann J, Macnaughton SJ, Kowalchuk GA, Haines JR, Kline E & White DC (2000) Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: effect of profiling method. *Journal of Microbiological Methods* **40**: 19-31.

Ciric L, Philp JC & Whiteley AS (2010) Hydrocarbon utilization within a diesel-degrading bacterial consortium. *FEMS Microbiology Letters* **303**: 116-122.

Cleary DF (2003) An examination of scale of assessment, logging and ENSO-induced fires on butterfly diversity in Borneo. *Oecologia* **135**: 313-321.

Cleary D, Oliveira V, Gomes N, Pereira A, Henriques I, Marques B, Almeida A, Cunha A, Correia A & Lillebø A (2012) Impact of sampling depth and plant species on local environmental conditions, microbiological parameters and bacterial composition in a mercury contaminated salt marsh. *Marine Pollution Bulletin* **64**: 263-271.

Cleary DF, Becking LE, Voogd NJ, Pires AC, Polónia AR, Egas C & Gomes N (2013) Habitat-and host-related variation in sponge bacterial symbiont communities in Indonesian waters. *FEMS Microbiology Ecology* **85**: 465-482.

Coelho FJ, Sousa S, Santos L, Santos AL, Almeida A, Gomes N & Cunha Â (2011) Exploring hydrocarbonoclastic bacterial communities in the estuarine surface microlayer. *Aquatic Microbial Ecology* **64**: 185-195.

Colmer TD & Flowers TJ (2008) Flooding tolerance in halophytes. *New Phytologist* **179**: 964-974.

Commendatore MG, Nievas ML, Amin O & Esteves JL (2012) Sources and distribution of aliphatic and polyaromatic hydrocarbons in coastal sediments from the Ushuaia Bay (Tierra del Fuego, Patagonia, Argentina). *Marine Environmental Research* **74**: 20-31.

Compant S, Duffy B, Nowak J, Clement C & Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* **71**: 4951-4959.

Compant S, van der Heijden MG & Sessitsch A (2010) Climate change effects on beneficial plant-microorganism interactions. *FEMS Microbiology Ecology* **73**: 197-214.

Córdova-Kreylos AL, Cao Y, Green PG, Hwang H-M, Kuivila KM, LaMontagne MG, Van De Werfhorst LC, Holden PA & Scow KM (2006) Diversity, composition, and geographical distribution of microbial communities in California salt marsh sediments. *Applied and Environmental Microbiology* **72**: 3357-3366.

Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Naeem S, Limburg K, Paruelo J & O'Neill RV (1997) The value of the world's ecosystem services and natural capital. *Nature* **387**: 253-260.

Coulon F, McKew BA, Osborn AM, McGenity TJ & Timmis KN (2007) Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environmental Microbiology* **9**: 177-186.

Coulon F, Chronopoulou P-M, Fahy A, Païssé S, Goñi-Urriza M, Peperzak L, Alvarez LA, McKew BA, Brussaard CP & Underwood GJ (2012) Hydrocarbon biodegradation in coastal mudflats: the central role of dynamic tidal biofilms dominated by aerobic

hydrocarbonoclastic bacteria and diatoms. *Applied and Environmental Microbiology* **78**: 3638-3648.

Couto M, Basto M & Vasconcelos M (2011) Suitability of different salt marsh plants for petroleum hydrocarbons remediation. *Chemosphere* **84**: 1052-1057.

Cravo-Laureau C, Matheron R, Cayol J-L, Joulian C & Hirschler-Réa A (2004) *Desulfatibacillum aliphaticivorans* gen. nov., sp. nov., an n-alkane-and n-alkene-degrading, sulfate-reducing bacterium. *Anglais* **54**: 77-83.

Cunha M, Pedro R, Almeida M & Silva M (2005) Activity and growth efficiency of heterotrophic bacteria in a salt marsh (Ria de Aveiro, Portugal). *Microbiological Research* **160**: 279-290.

Cunliffe M & Kertesz MA (2006) Effect of *Sphingobium yanoikuyae* B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environmental Pollution* **144**: 228-237.

Cunningham SD, Anderson TA, Schwab AP & Hsu F (1996) Phytoremediation of soils contaminated with organic pollutants. *Advances in Agronomy* **56**: 55-114.

Curado G, Rubio-Casal A, Figueroa E & Castillo J (2014) Potential of Spartina maritima in restored salt marshes for phytoremediation of metals in a highly polluted estuary. *International Journal of Phytoremediation* **16**: 1209-1220

Daane LL, Harjono I, Zylstra GJ & Haggblom MM (2001) Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Applied and Environmental Microbiology* **67**: 2683-2691.

Das N & Chandran P (2010) Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology Research International* **2011**: 1-13.

Dennis JJ (2005) The evolution of IncP catabolic plasmids. *Current Opinion in Biotechnology* **16**: 291-298.

Dias JM, Lopes J & Dekeyser I (1999) Hydrological characterisation of Ria de Aveiro, Portugal, in early summer. *Oceanologica Acta* **22**: 473-485.

Diaz MP, Grigson SJ, Peppiatt CJ & Burgess JG (2000) Isolation and characterization of novel hydrocarbon-degrading euryhaline consortia from crude oil and mangrove sediments. *Marine Biotechnology* **2**: 522-532.

Díez S, Jover E, Bayona JM & Albaigés J (2007) Prestige oil spill. III. Fate of a heavy oil in the marine environment. *Environmental Science & Technology* **41**: 3075-3082.

Ding GC, Heuer H, Zuhlke S, Spiteller M, Pronk GJ, Heister K, Kogel-Knabner I & Smalla K (2010) Soil type-dependent responses to phenanthrene as revealed by determining the diversity and abundance of polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase genes by using a novel PCR detection system. *Applied and Environmental Microbiology* **76**: 4765-4771.

Domingues PM, Louvado A, Oliveira V, Coelho FJ, Almeida A, Gomes NC & Cunha A (2013) Selective cultures for the isolation of biosurfactant producing bacteria: comparision of different combination of enviromentla inocula and hydrophobic carbon sources. *Preparative Biochemistry and Biotechnology* **43**: 237-255.

Dubinsky EA, Conrad ME, Chakraborty R, Bill M, Borglin SE, Hollibaugh JT, Mason OU, M. Piceno Y, Reid FC & Stringfellow WT (2013) Succession of hydrocarbon-degrading bacteria in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. *Environmental Science & Technology* **47**: 10860-10867.

Escalante-Espinosa E, Gallegos-Martínez M, Favela-Torres E & Gutiérrez-Rojas M (2005) Improvement of the hydrocarbon phytoremediation rate by *Cyperus laxus* Lam. inoculated with a microbial consortium in a model system. *Chemosphere* **59**: 405-413.

EPA U (2000) Introduction to Phytoremediation. EPA-report EPA/600/R-99/107. http://clu-in.org/techpubs.htm. Accessed May 2000.

Ferrera-Rodríguez O, Greer CW, Juck D, Consaul L, Martínez-Romero E & Whyte L (2013) Hydrocarbon-degrading potential of microbial communities from Arctic plants. *Journal of Applied Microbiology* **114**: 71-83.

Food and Agriculture Organization of the United Nations (FAOUN)(1984) Physical and chemical methods of soil and water analysis. *Soils Bulletin* **10**:1-275.

Fitzgerald E, Caffrey J, Nesaratnam S & McLoughlin P (2003) Copper and lead concentrations in salt marsh plants on the Suir Estuary, Ireland. *Environmental Pollution* **123**: 67-74.

Flocco CG, Gomes NC, Mac Cormack W & Smalla K (2009) Occurrence and diversity of naphthalene dioxygenase genes in soil microbial communities from the Maritime Antarctic. *Environmental Microbiology* **11**: 700-714.

Flowers TJ & Colmer TD (2008) Salinity tolerance in halophytes. *The New phytologist* **179**: 945-963.

Fox WM, Connor L, Copplestone D, Johnson MS & Leah RT (2001) The organochlorine contamination history of the Mersey estuary, UK, revealed by analysis of sediment cores from salt marshes. *Marine Environmental Research* **51**: 213-227.

Freeman DJ & Cattell FC (1990) Woodburning as a source of atmospheric polycyclic aromatic hydrocarbons. *Environmental Science & Technology* **24**: 1581-1585.

Frick C, Germida J & Farrell R (1999) Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. In: *Technical Seminar on Chemical Spillsp,* pp. 105a-124a. Environment Canada.

Gao Y & Zhu L (2004) Plant uptake, accumulation and translocation of phenanthrene and pyrene in soils. *Chemosphere* **55**: 1169-1178.

Garbisu C & Alkorta I (2001) Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. *Bioresource Technology* **77**: 229-236.

Geiselbrecht, DA, Herwig, PR, Deming, WJ & Staley TJ (1996) Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediments. *Applied and Environmental Microbiology* **62**: 3344–3349.

Genouw G, De Naeyer F, Van Meenen P, Van de Werf H, De Nijs W & Verstraete W (1994) Degradation of oil sludge by landfarming - a case-study at the Ghent harbour. *Biodegradation* **5**: 37-46.

Genty B, Harbinson J & Baker N (1990) Relative quantum efficiencies of the two photosystems of leaves in photorespiratory and non-respiratory conditions. *Plant Physiology and Biochemistry* **28**: 1-10.

Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore FP & Moore ER (2004) Colonisation of poplar trees by *gfp* expressing bacterial endophytes. *FEMS Microbiology Ecology* **48**: 109-118.

Germaine KJ, Liu X, Cabellos GG, Hogan JP, Ryan D & Dowling DN (2006) Bacterial endophyte-enhanced phytoremediation of the organochlorine herbicide 2, 4-dichlorophenoxyacetic acid. *FEMS Microbiology Ecology* **57**: 302-310.

Germaine KJ, Keogh E, Ryan D & Dowling DN (2009) Bacterial endophyte-mediated naphthalene phytoprotection and phytoremediation. *FEMS Microbiology Letters* **296**: 226-234.

Gioia R, Steinnes E, Thomas GO, Mejier SN & Jones KC (2006) Persistent organic pollutants in European background air: derivation of temporal and latitudinal trends. *Journal of Environmental Monitoring* **8**: 700-710.

Gogou AI, Apostolaki M & Stephanou EG (1998) Determination of organic molecular markers in marine aerosols and sediments: one-step flash chromatography compound

class fractionation and capillary gas chromatographic analysis. *Journal of Chromatography A* **799**: 215-231.

Gomes NC, Kosheleva IA, Abraham WR & Smalla K (2005) Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. *FEMS Microbiology Ecology* **54**: 21-33.

Gomes NC, Borges LR, Paranhos R, Pinto FN, Krogerrecklenfort E, Mendonca-Hagler LC & Smalla K (2007) Diversity of *ndo* genes in mangrove sediments exposed to different sources of polycyclic aromatic hydrocarbon pollution. *Applied and Environmental Microbiology* **73**: 7392-7399.

Gomes NC, Borges LR, Paranhos R, Pinto FN, Mendonca-Hagler LC & Smalla K (2008) Exploring the diversity of bacterial communities in sediments of urban mangrove forests. *FEMS Microbiology Ecology* **66**: 96-109.

Gomes NC, Flocco CG, Costa R, Junca H, Vilchez R, Pieper DH, Krogerrecklenfort E, Paranhos R, Mendonca-Hagler LC & Smalla K (2010a) Mangrove microniches determine the structural and functional diversity of enriched petroleum hydrocarbon-degrading consortia. *FEMS Microbiology Ecology* **74**: 276-290.

Gomes NC, Cleary DF, Pinto FN, Egas C, Almeida A, Cunha A, Mendonça-Hagler LC & Smalla K (2010b) Taking root: enduring effect of rhizosphere bacterial colonization in mangroves. *PloS one* **5**: e14065.

Gomes N, Manco SC, Pires ACC, Gonçalves SF, Calado R, Cleary DF & Loureiro S (2013) Richness and composition of sediment bacterial assemblages in an Atlantic port environment. *Science of The Total Environment* **452**: 172-180.

Gomes N, Cleary DF, Pires AC, Almeida A, Cunha A, Mendonça-Hagler L & Smalla K (2014) Assessing variation in bacterial composition between the rhizospheres of two mangrove tree species. *Estuarine, Coastal and Shelf Science* **139**: 40-45.

Gonul LT & Kucuksezgin F (2012) Aliphatic and polycyclic aromatic hydrocarbons in the surface sediments from the Eastern Aegean: assessment and source recognition of petroleum hydrocarbons. *Environmental Science and Pollution Research* **19**: 31-41.

Grayston SJ, Vaughan D & Jones D (1996) Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. *Applied Soil Ecology* **5**: 29-56.

Günther T, Dornberger U & Fritsche W (1996) Effects of ryegrass on biodegradation of hydrocarbons in soil. *Chemosphere* **33**: 203-215.

Guo W, He M, Yang Z, Lin C & Quan X (2011) Aliphatic and polycyclic aromatic hydrocarbons in the Xihe River, an urban river in China's Shenyang City: Distribution and risk assessment. *Journal of Hazardous Materials* **186**: 1193-1199.

Gurska J, Wang W, Gerhardt KE, Khalid AM, Isherwood DM, Huang X-D, Glick BR & Greenberg BM (2009) Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. *Environmental Science & Technology* **43**: 4472-4479.

Habe H & Omori T (2003) Genetics of Polycyclic Aromatic Hydrocarbon Metabolism in Diverse Aerobic Bacteria. *Bioscience, Biotechnology, and Biochemistry* **67**: 225-243.

Haritash A & Kaushik C (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *Journal of Hazardous Materials* **169**: 1-15.

Harwati TU, Kasai Y, Kodama Y, Susilaningsih D & Watanabe K (2007) Characterization of diverse hydrocarbon-degrading bacteria isolated from Indonesian seawater. *Microbes and Environments* **22**: 412-415.

Herrick BJ, Stuart-Keil GK, Ghiorse CW & Madsen LE (1997) Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. *Applied and Environmental Microbiology* **63**: 2330-2337.

Heuer H, Krsek M, Baker P, Smalla K & Wellington E (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63**: 3233-3241.

Heuer H, Wieland G, Schönfeld J, Schönwälder A, Gomes N & Smalla K (2001) Bacterial community profiling using DGGE or TGGE analysis. *Environmental molecular microbiology: Protocols and Applications* **9**: 177-190.

Hines ME, Evans RS, Willis SG, Rooney-Varga JN, Genthner BRS, Friedman S & Devereux R (1999) Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the rhizosphere of *Spartina alterniflora*. *Applied and Environmental Microbiology* **65**: 2209-2216.

Hong SH, Ryu H, Kim J & Cho K-S (2011) Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp. S2RP-17. *Biodegradation* **22**: 593-601.

Hung GA & Chmura GL (2006) Mercury accumulation in surface sediments of salt marshes of the Bay of Fundy. *Environmental Pollution* **142**: 418-431.

Hwang H-M, Green PG & Young TM (2006) Tidal salt marsh sediment in California, USA. Part 1: occurrence and sources of organic contaminants. *Chemosphere* **64**: 1383-1392.

Iqbal J, Gisclair D, McMillin DJ & Portier RJ (2007) Aspects of petrochemical pollution in southeastern Louisiana (USA): Pre-Katrina background and source characterization. *Environmental Toxicology and Chemistry* **26**: 2001-2009.

Izmalkova TY, Sazonova OI, Sokolov SL, Kosheleva IA & Boronin AM (2005) Diversity of genetic systems responsible for naphthalene biodegradation in *Pseudomonas fluorescens* strains. *Microbiology* **74**: 60-68.

Juhanson J, Truu J, Heinaru E & Heinaru A (2009) Survival and catabolic performance of introduced *Pseudomonas* strains during phytoremediation and bioaugmentation field experiment. *FEMS Microbiology Ecology* **70**: 446-455.

Jung H, Sohn K-D, Neppolian B & Choi H (2008) Effect of soil organic matter (SOM) and soil texture on the fatality of indigenous microorganisms in intergrated ozonation and biodegradation. *Journal of Hazardous Materials* **150**: 809-817.

Kaakinen J, Vä P, Kuokkanen T & Roppola K (2007) Studies on the effects of certain soil properties on the biodegradation of oils determined by the manometric respirometric method. *Journal of Analytical Methods in Chemistry* **2007:** 1-7.

Khan S, Afzal M, Iqbal S & Khan QM (2013) Plant–bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere* **90**: 1317-1332.

Kim GB, Maruya KA, Lee RF, Lee J-H, Koh C-H & Tanabe S (1999) Distribution and sources of polycyclic aromatic hydrocarbons in sediments from Kyeonggi Bay, Korea. *Marine Pollution Bulletin* **38**: 7-15.

Kim S-J & Kwon K (2010) Marine, hydrocarbon-degrading Alphaproteobacteria. In: *Handbook of hydrocarbon and lipid microbiology*, pp. 1707-1714. Springer.

Kouridaki I, Polymenakou PN, Tselepides A, Mandalakis M & Smith Jr KL (2010) Phylogenetic diversity of sediment bacteria from the deep Northeastern Pacific Ocean: a comparison with the deep Eastern Mediterranean Sea. *International Microbiology* **13**: 143-150.

Kubota M, Nodate M, Yasumoto-Hirose M, Uchiyama T, Kagami O, Shizuri Y & Misawa N (2005) Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments. *Biosci Biotechnol Biochem* **69**: 2421-2430.

Kuiper I, Lagendijk EL, Bloemberg GV & Lugtenberg BJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. *Molecular Plant-Microbe Interactions* **17**: 6-15.

Labbé D, Margesin R, Schinner F, Whyte LG & Greer CW (2007) Comparative phylogenetic analysis of microbial communities in pristine and hydrocarbon-contaminated Alpine soils. *FEMS Microbiology Ecology* **59**: 466-475.

Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV & Knight R (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology* **31**: 814-821.

Langworthy DE, Stapleton RD, Sayler GS & Findlay RH (1998) Genotypic and phenotypic responses of a riverine microbial community to polycyclic aromatic hydrocarbon contamination. *Applied and Environmental Microbiology* **64**: 3422-3428.

Lauber CL, Hamady M, Knight R & Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology* **75**: 5111-5120.

Leahy JG & Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews* **54**: 305-315.

Lee S & Cundy A (2001) Heavy metal contamination and mixing processes in sediments from the Humber Estuary, Eastern England. *Estuarine, Coastal and Shelf Science* **53**: 619-636.

Legendre P & Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. *Oecologia* **129**: 271-280.

Liang Y, Van Nostrand JD, Deng Y, He Z, Wu L, Zhang X, Li G & Zhou J (2010) Functional gene diversity of soil microbial communities from five oil-contaminated fields in China. *The ISME Journal* **5**: 403-413.

Lin Q & Mendelssohn IA (1996) A comparative investigation of the effects of south Louisiana crude oil on the vegetation of fresh, brackish and salt marshes. *Marine Pollution Bulletin* **32**: 202-209.

Lin Q & Mendelssohn IA (1998) The combined effects of phytoremediation and biostimulation in enhancing habitat restoration and oil degradation of petroleum contaminated wetlands. *Ecological Engineering* **10**: 263-274.

Lin Q & Mendelssohn IA (2009) Potential of restoration and phytoremediation with *Juncus roemerianus* for diesel-contaminated coastal wetlands. *Ecological Engineering* **35**: 85-91.

Lindgren JF, Hassellöv I-M & Dahllöf I (2014) PAH effects on meio-and microbial benthic communities strongly depend on bioavailability. *Aquatic Toxicology* **146**: 230-238.

Liu K, Han W, Pan W-P & Riley JT (2001) Polycyclic aromatic hydrocarbon (PAH) emissions from a coal-fired pilot FBC system. *Journal of Hazardous Materials* **84**: 175-188.

Liu W, Sun J, Ding L, Luo Y, Chen M & Tang C (2013) Rhizobacteria (*Pseudomonas* sp. SB) assist phytoremediation of oily-sludge-contaminated soil by tall fescue (*Testuca arundinacea* L.). *Plant and Soil* **371**: 533-542.

Liu X, Wang Z, Zhang X, Wang J, Xu G, Cao Z, Zhong C & Su P (2011) Degradation of diesel-originated pollutants in wetlands by *Scirpus triqueter* and microorganisms. *Ecotoxicology and Environmental Safety* **74**: 1967-1972.

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M & der Lelie Dv (2002) Endophytic Bacteria and Their Potential Applications. *Critical Reviews in Plant Sciences* **21**: 583-606.

Luo X, Chen S, Mai B, Zeng Y, Sheng G & Fu J (2005) Distribution and sources of polycyclic aromatic hydrocarbons in sediments from rivers of Pearl River Delta and its nearby South China Sea. *Environmental Science* **26**: 129-134.

Ma Y, Wang L & Shao Z (2006) *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. *Environmental Microbiology* **8**: 455-465.

Ma B, He Y, Chen H-h, Xu J-m & Rengel Z (2010) Dissipation of polycyclic aromatic hydrocarbons (PAHs) in the rhizosphere: Synthesis through meta-analysis. *Environmental Pollution* **158**: 855-861.

Magi E, Bianco R, Ianni C & Di Carro M (2002) Distribution of polycyclic aromatic hydrocarbons in the sediments of the Adriatic Sea. *Environmental Pollution* **119**: 91-98.

Mahajan MC, Phale PS & Vaidyanathan CS (1994) Evidence for the involvement of multiple pathways in the biodegradation of 1-and 2-methylnaphthalene by *Pseudomonas putida* CSV86. *Archives of Microbiology* **161**: 425-433.

Manousaki E & Kalogerakis N (2011) Halophytes - an emerging trend in phytoremediation. *International Journal of Phytoremediation* **13**: 959-969.

Mao J, Luo Y, Teng Y & Li Z (2012) Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes. *International Biodeterioration & Biodegradation* **70**: 141-147.

Marques B, Lillebø A, Pereira E & Duarte A (2011) Mercury cycling and sequestration in salt marshes sediments: An ecosystem service provided by *Juncus maritimus* and *Scirpus maritimus*. *Environmental Pollution* **159**: 1869-1876.

Marschner P, Yang C-H, Lieberei R & Crowley DE (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry* **33**: 1437-1445.

Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P, Prudhomme M, Alloing G & Hakenbeck R (1992) A highly conserved repeated DNA element located in the chromosome of Streptococcus pneumoniae. *Nucleic Acids Research* **20**: 3479-3483.

Martins M, Ferreira AM & Vale C (2008) The influence of Sarcocornia fruticosa on retention of PAHs in salt marsh sediments (Sado estuary, Portugal). *Chemosphere* **71**: 1599-1606.

McGee BL, Fisher DJ, Yonkos LT, Ziegler GP & Turley S (1999) Assessment of sediment contamination, acute toxicity, and population viability of the estuarine amphipod *Leptocheirus plumulosus* in Baltimore Harbor, Maryland, USA. *Environmental Toxicology and Chemistry* **18**: 2151-2160.

McGenity TJ (2014) Hydrocarbon biodegradation in intertidal wetland sediments. *Current Opinion in Biotechnology* **27**: 46-54.

Mendelssohn IA, Hester MW, Sasser C & Fischel M (1990) The effect of a Louisiana crude oil discharge from a pipeline break on the vegetation of a southeast Louisiana brackish marsh. *Oil and Chemical Pollution* **7**: 1-15.

Mergeay M, Nies D, Schlegel H, Gerits J, Charles P & Van Gijsegem F (1985) Alcaligenes eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *Journal of Bacteriology* **162**: 328-334.

Meudec A, Dussauze J, Deslandes E & Poupart N (2006) Evidence for bioaccumulation of PAHs within internal shoot tissues by a halophytic plant artificially exposed to petroleum-polluted sediments. *Chemosphere* **65**: 474-481.

Militon C, Boucher D, Vachelard C, Perchet G, Barra V, Troquet J, Peyretaillade E & Peyret P (2010) Bacterial community changes during bioremediation of aliphatic hydrocarbon-contaminated soil. *FEMS Microbiology Ecology* **74**: 669-681.

Mille G, Almallah M, Bianchi M, Van Wambeke F & Bertrand J (1991) Effect of salinity on petroleum biodegradation. *Fresenius' Journal of Analytical Chemistry* **339**: 788-791.

Mille G, Asia L, Guiliano M, Malleret L & Doumenq P (2007) Hydrocarbons in coastal sediments from the Mediterranean sea (Gulf of Fos area, France). *Marine Pollution Bulletin* **54**: 566-575.

Minai-Tehrani D, Minoui S & Herfatmanesh A (2009) Effect of salinity on biodegradation of polycyclic aromatic hydrocarbons (PAHs) of heavy crude oil in soil. *Bulletin of Environmental Contamination and Toxicology* **82**: 179-184.

Mishra S, Jyot J, Kuhad RC & Lal B (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Applied and Environmental Microbiology* **67**: 1675-1681.

Mitsch WJ & Gosselink JG (2000) The value of wetlands: importance of scale and landscape setting. *Ecological Economics* **35**: 25-33.

Moreira IT, Oliveira O, Triguis JA, dos Santos AM, Queiroz AF, Martins C, Silva CS & Jesus RS (2011) Phytoremediation using *Rizophora mangle* L. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's). *Microchemical Journal* **99**: 376-382.

Moreira SM, Lima I, Ribeiro R & Guilhermino L (2006) Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and in situ assays. *Aquatic Toxicology* **78**: 186-201.

Mucha AP, Almeida CMR, Bordalo AA & Vasconcelos M (2010) LMWOA (low molecular weight organic acid) exudation by salt marsh plants: natural variation and response to Cu contamination. *Estuarine, Coastal and Shelf Science* **88**: 63-70.

Mucha AP, Almeida CMR, Magalhães CM, Vasconcelos M & Bordalo AA (2011) Salt marsh plant–microorganism interaction in the presence of mixed contamination. *International Biodeterioration & Biodegradation* **65**: 326-333.

Muratova AY, Bondarenkova A, Panchenko L & Turkovskaya O (2010) Use of integrated phytoremediation for cleaning-up of oil-sludge-contaminated soil. *Applied Biochemistry and Microbiology* **46**: 789-794.

Nedbal L, Trtìlek M & Herppich W (2000) Methods and equipment for fluorescence imaging on plant material. *Bornimer Agrartechnische Berichte* **25**: 127-135.

Newton RJ, Jones SE, Eiler A, McMahon KD & Bertilsson S (2011) A guide to the natural history of freshwater lake bacteria. *Microbiology and Molecular Biology Reviews* **75**: 14-49.

Ni Chadhain SM, Norman RS, Pesce KV, Kukor JJ & Zylstra GJ (2006) Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. *Applied and Environmental Microbiology* **72**: 4078-4087.

O'Day PA, Carroll SA, Randall S, Martinelli RE, Anderson SL, Jelinski J & Knezovich JP (2000) Metal speciation and bioavailability in contaminated estuary sediments, Alameda Naval Air Station, California. *Environmental Science & Technology* **34**: 3665-3673.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'Hara R, Simpson G, Solymos P, Henry M & Stevens H (2011) vegan: Community Ecology Package. *R package*. http://www.r-project.org.

Oliveira AAd & Mendelssohn IA (2009) Potential of restoration and phytoremediation with *Juncus roemerianus* for diesel-contaminated coastal wetlands. *Ecological Engineering* **35**: 85-91.

Oliveira V, Santos AL, Coelho F, Gomes NC, Silva H, Almeida A & Cunha A (2010) Effects of monospecific banks of salt marsh vegetation on sediment bacterial communities. *Microbial Ecology* **60**: 167-179.

Oliveira V, Santos AL, Aguiar C, Santos L, Salvador ÂC, Gomes N, Silva H, Rocha SM, Almeida A & Cunha Â (2012) Prokaryotes in salt marsh sediments of Ria de

Aveiro: Effects of halophyte vegetation on abundance and diversity. *Estuarine, Coastal and Shelf Science* **110**: 61-68.

Oliveira V, Gomes N, Almeida A, Silva A, Simões MM, Smalla K & Cunha Â (2014) Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria. *Molecular Ecology* **23**: 1392-1404.

Ono A, Miyazaki R, Sota M, Ohtsubo Y, Nagata Y & Tsuda M (2007) Isolation and characterization of naphthalene-catabolic genes and plasmids from oil-contaminated soil by using two cultivation-independent approaches. *Applied Microbiology and Biotechnology* **74**: 501-510.

Pacheco M & Santos MA (2001) Biotransformation, Endocrine, and Genetic Responses of *Anguilla anguilla* L. to Petroleum Distillate Products and Environmentally Contaminated Waters. *Ecotoxicology and Environmental Safety* **49**: 64-75.

Paisse S, Coulon F, Goñi-Urriza M, Peperzak L, McGenity TJ & Duran R (2008) Structure of bacterial communities along a hydrocarbon contamination gradient in a coastal sediment. *FEMS Microbiology Ecology* **66**: 295-305.

Park JW & Crowley DE (2006) Dynamic changes in nahAc gene copy numbers during degradation of naphthalene in PAH-contaminated soils. *Applied Microbiology and Biotechnology* **72**: 1322-1329.

Patel V, Cheturvedula S & Madamwar D (2012) Phenanthrene degradation by *Pseudoxanthomonas* sp. DMVP2 isolated from hydrocarbon contaminated sediment of Amlakhadi canal, Gujarat, India. *Journal of Hazardous Materials* **201**: 43-51.

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS & Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiology Reviews* **32**: 927-955.

Pezeshki S, Hester M, Lin Q & Nyman J (2000) The effects of oil spill and clean-up on dominant US Gulf coast marsh macrophytes: a review. *Environmental Pollution* **108**: 129-139.

Phillips LA, Greer CW & Germida JJ (2006) Culture-based and culture-independent assessment of the impact of mixed and single plant treatments on rhizosphere microbial communities in hydrocarbon contaminated flare-pit soil. *Soil Biology and Biochemistry* **38**: 2823-2833.

Phillips LA, Germida JJ, Farrell RE & Greer CW (2008) Hydrocarbon degradation potential and activity of endophytic bacteria associated with prairie plants. *Soil Biology and Biochemistry* **40**: 3054-3064.

Phillips LA, Greer CW, Farrell RE & Germida JJ (2012) Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil. *Applied Soil Ecology* **52**: 56-64.

Pilon-Smits E (2005) Phytoremediation. *Annual Review of Plant Biology* **56**: 15-39.

Pires AC, Cleary DF, Almeida A, Cunha Â, Dealtry S, Mendonça-Hagler LC, Smalla K & Gomes NC (2012) Denaturing gradient gel electrophoresis and barcoded pyrosequencing reveal unprecedented archaeal diversity in mangrove sediment and rhizosphere samples. *Applied and Environmental Microbiology* **78**: 5520-5528.

Polónia AR, Cleary DF, Duarte LN, de Voogd NJ & Gomes NC (2014) Composition of Archaea in Seawater, Sediment, and Sponges in the Kepulauan Seribu Reef System, Indonesia. *Microbial Ecology* 1-15.

Polymenakou PN, Bertilsson S, Tselepides A & Stephanou EG (2005) Bacterial community composition in different sediments from the Eastern Mediterranean Sea: a comparison of four 16S ribosomal DNA clone libraries. *Microbial Ecology* **50**: 447-462.

Pucci O, Bak M, Peressutti S, Klein I, Härtig C, Alvarez H & Wünsche L (2000) Influence of crude oil contamination on the bacterial community of semiarid soils of Patagonia (Argentina). *Acta Biotechnologica* **20**: 129-146.

Quintino V, Rodrigues A & Gentil F (1989) Assessment of macrozoobenthic communities in the lagoon of Óbidos, western coast of Portugal. *Scientia Marina* **53**: 645-654.

Radwan S, Dashti N & El-Nemr I (2005) Enhancing the growth of *Vicia faba* plants by microbial inoculation to improve their phytoremediation potential for oily desert areas. *International Journal of Phytoremediation* **7**: 19-32.

RamanaRao MV, Weindorf D, Breitenbeck G & Baisakh N (2012) Differential expression of the transcripts of *Spartina alterniflora* Loisel (smooth cordgrass) induced in response to petroleum hydrocarbon. *Molecular Biotechnology* **51**: 18-26.

Readman J, Fillmann G, Tolosa I, Bartocci J, Villeneuve J-P, Catinni C & Mee L (2002) Petroleum and PAH contamination of the Black Sea. *Marine Pollution Bulletin* **44**: 48-62.

Reboreda R & Cacador I (2007) Halophyte vegetation influences in salt marsh retention capacity for heavy metals. *Environmental Pollution* **146**: 147-154.

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

Ribeiro H, Mucha AP, Marisa R Almeida C & Bordalo AA (2013) Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh. *Science of The Total Environment* **458**: 568-576.

Riis V, Kleinsteuber S & Babel W (2003) Influence of high salinities on the degradation of diesel fuel by bacterial consortia. *Canadian Journal of Microbiology* **49**: 713-721.

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AK, Kent AD, Daroub SH, Camargo FA, Farmerie WG & Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal* **1**: 283-290.

Rosenblueth M & Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions* **19**: 827-837.

Rosso PH, Pushnik JC, Lay M & Ustin SL (2005) Reflectance properties and physiological responses of *Salicornia virginica* to heavy metal and petroleum contamination. *Environmental Pollution* **137**: 241-252.

Rubio-Casal A, Castillo J, Luque C & Figueroa M (2003) Influence of salinity on germination and seeds viability of two primary colonizers of Mediterranean salt pans. *Journal of Arid Environments* **53**: 145-154.

Ryan RP, Germaine K, Franks A, Ryan DJ & Dowling DN (2008) Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters* **278**: 1-9.

Samanta SK, Singh OV & Jain RK (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology* **20**: 243-248.

Sambrook J & Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York.

Sanger D, Holland A & Scott G (1999) Tidal creek and salt marsh sediments in South Carolina coastal estuaries: II. Distribution of organic contaminants. *Archives of Environmental Contamination and Toxicology* **37**: 458-471.

Santos HF, Carmo FL, Paes JE, Rosado AS & Peixoto RS (2011) Bioremediation of mangroves impacted by petroleum. *Water, Air, & Soil Pollution* **216**: 329-350.

Santos L, Cunha A, Silva H, Cacador I, Dias JM & Almeida A (2007) Influence of salt marsh on bacterial activity in two estuaries with different hydrodynamic characteristics (Ria de Aveiro and Tagus Estuary). *FEMS Microbiology Ecology* **60**: 429-441.

Schippers A, Bosecker K, Spröer C & Schumann P (2005) *Microbacterium oleivorans* sp. nov. and *Microbacterium hydrocarbonoxydans* sp. nov., novel crude-oil-degrading Gram-positive bacteria. *Anglais* **55**: 655-660.

Schneegurt MA & Kulpa CF (1998) The application of molecular techniques in environmental biotechnology for monitoring microbial systems. *Biotechnology and Applied Biochemistry* **27**: 73-79.

Schubert P, Fernández-Escobar I, Rosenberg E & Bayona J-M (1998) Evaluation of desulfurization procedures for the elimination of sulfur interferences in the organotin analysis of sediments. *Journal of Chromatography A* **810**: 245-251.

Segura A, Rodriguez-Conde S, Ramos C & Ramos JL (2009) Bacterial responses and interactions with plants during rhizoremediation. *Microbial Biotechnology* **2**: 452-464.

Shaw LJ & Burns RG (2003) Biodegradation of organic pollutants in the rhizosphere. *Advances in Applied Microbiology* **53**: 1-60.

Sheng X & Gong J (2006) Increased degradation of phenanthrene in soil by *Pseudomonas* sp. GF3 in the presence of wheat. *Soil Biology and Biochemistry* **38**: 2587-2592.

Siciliano SD & Germida JJ (1998) Degradation of chlorinated benzoic acid mixtures by plant–bacteria associations. *Environmental Toxicology and Chemistry* **17**: 728-733.

Siciliano SD, Fortin N, Mihoc A, *et al.* (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied and Environmental Microbiology* **67**: 2469-2475.

Siciliano SD, Germida JJ, Banks K & Greer CW (2002) Changes in Microbial Community Composition and Function during a Polyaromatic Hydrocarbon Phytoremediation Field Trial. *Applied and Environmental Microbiology* **69**: 483-489.

Silva H, Dias J & Caçador I (2009) Is the salt marsh vegetation a determining factor in the sedimentation processes? *Hydrobiologia* **621**: 33-47.

Simpson CD, Mosi AA, Cullen WR & Reimer KJ (1996) Composition and distribution of polycyclic aromatic hydrocarbon contamination in surficial marine sediments from Kitimat Harbor, Canada. *Science of The Total Environment* **181**: 265-278.

Singh BK, Millard P, Whiteley AS & Murrell JC (2004) Unravelling rhizosphere-microbial interactions: opportunities and limitations. *Trends in Microbiology* **12**: 386-393.

Singleton DR, Richardson SD & Aitken MD (2011) Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. *Biodegradation* **22**: 1061-1073.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, & Tietze E (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Applied and Environmental Microbiology* **66**: 4854–4862.

Soil and Plant Analysis Council (SPAC) (2000) *Handbook of reference Methods*. CRC Press, Boca Raton, Florida

Soclo H, Garrigues P & Ewald M (2000) Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Cotonou (Benin) and Aquitaine (France) areas. *Marine Pollution Bulletin* **40**: 387-396.

Steeghs M, Bais HP, de Gouw J, Goldan P, Kuster W, Northway M, Fall R & Vivanco JM (2004) Proton-transfer-reaction mass spectrometry as a new tool for real time

analysis of root-secreted volatile organic compounds in Arabidopsis. *Plant Physiology* **135**: 47-58.

Suárez-Suárez A, López-López A, Tovar-Sánchez A, Yarza P, Orfila A, Terrados J, Arnds J, Marqués S, Niemann H & Schmitt-Kopplin P (2011) Response of sulfate-reducing bacteria to an artificial oil-spill in a coastal marine sediment. *Environmental Microbiology* **13**: 1488-1499.

Sutton NB, Maphosa F, Morillo JA, Al-Soud WA, Langenhoff AA, Grotenhuis T, Rijnaarts HH & Smidt H (2013) Impact of long-term diesel contamination on soil microbial community structure. *Applied and Environmental Microbiology* **79**: 619-630.

Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J & van der Lelie D (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. *Applied and Environmental Microbiology* **71**: 8500-8505.

Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J & van der Lelie D (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. *Applied and Environmental Microbiology* **75**: 748-757.

Tam N, Ke L, Wang X & Wong Y (2001) Contamination of polycyclic aromatic hydrocarbons in surface sediments of mangrove swamps. *Environmental Pollution* **114**: 255-263.

Team RDC (2013) R: a language and enviroment for statistical computing. R foundation for statistical computing, Viena, Austria, Version 2.15.

Todd GD, Chessin RL & Colman J (1999) Toxicological profile for total petroleum hydrocarbons (TPH). *Atlanta: Department of Health and Human Services Agency for Toxic Substances and Disease Registry*. http://stacks.cdc.gov/view/cdc/6416/EC9.47-EC9.84

Top EM, Springael D & Boon N (2002) Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *FEMS Microbiology Ecology* **42**: 199-208.

Top EM & Springael D (2003) The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Current Opinion in Biotechnology* **14**: 262-269.

Trapp S & Karlson U (2001) Aspects of phytoremediation of organic pollutants. *Journal of Soils and Sediments* **1**: 37-43.

Valega M, Lillebo AI, Pereira ME, Cacador I, Duarte AC & Pardal MA (2008) Mercury in salt marshes ecosystems: *Halimione portulacoides* as biomonitor. *Chemosphere* **73**: 1224-1229.

Valega M, Lima AI, Figueira EM, Pereira E, Pardal MA & Duarte AC (2009) Mercury intracellular partitioning and chelation in a salt marsh plant, *Halimione portulacoides* (L.) Aellen: strategies underlying tolerance in environmental exposure. *Chemosphere* **74**: 530-536.

Valiela I, Rutecki D & Fox S (2004) Salt marshes: biological controls of food webs in a diminishing environment. *Journal of Experimental Marine Biology and Ecology* **300**: 131-159.

Vane C, Harrison I & Kim A (2007) Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in sediments from the Mersey Estuary, UK. *Science of The Total Environment* **374**: 112-126.

Vazquez S, Nogales B, Ruberto L, Hernandez E, Christie-Oleza J, Lo Balbo A, Bosch R, Lalucat J & Mac Cormack W (2009) Bacterial community dynamics during bioremediation of diesel oil-contaminated Antarctic soil. *Microbial Ecology* **57**: 598-610.

Venosa AD & Zhu X (2003) Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. *Spill Science & Technology Bulletin* **8**: 163-178.

Vernberg FJ (1993) Salt-marsh processes: A Review. *Environmental Toxicology and Chemistry* **12**: 2167-2195.

Viñas M, Sabaté J, Espuny MJ & Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Applied and Environmental Microbiology* **71**: 7008-7018.

Waisel Y (2012) Biology of halophytes. Elsevier.

Wang X-C, Zhang Y-X & Chen RF (2001) Distribution and partitioning of polycyclic aromatic hydrocarbons (PAHs) in different size fractions in sediments from Boston Harbor, United States. *Marine Pollution Bulletin* **42**: 1139-1149.

Wang Y & Qian P-Y (2009) Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. *PLoS ONE* **4**: e7401.

Wang Y-F & Tam NF-Y (2012) Natural attenuation of contaminated marine sediments from an old floating dock Part II: Changes of sediment microbial community structure and its relationship with environmental variables. *Science of The Total Environment* **423**: 95-103.

Wang Y, Sheng H-F, He Y, Wu J-Y, Jiang Y-X, Tam NF-Y & Zhou H-W (2012) Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Applied and Environmental Microbiology* **78**: 8264-8271.

Watts AW, Ballestero TP & Gardner KH (2006) Uptake of polycyclic aromatic hydrocarbons (PAHs) in salt marsh plants *Spartina alterniflora* grown in contaminated sediments. *Chemosphere* **62**: 1253-1260.

Watts AW, Ballestero TP & Gardner KH (2008) Soil and Atmospheric Inputs to PAH Concentrations in Salt Marsh Plants. *Water, Air, and Soil Pollution* **189**: 253-263.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Wick AF, Haus NW, Sukkariyah BF, Haering KC & Daniels WL (2011) Remediation of PAH-contaminated soils and sediments: A literature review. *Virginia Polytechnic Institute and State University*.
http://landrehab.org/userfiles/files/Dredge/Virginia%20Tech%20PAH%20Remediation%20Lit%20Review%202011.pdf

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H & Omori T (2002) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. *Applied Microbiology and Biotechnology* **58**: 202-209.

Wilson MS, Bakermans C & Madsen EL (1999) In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Applied and Environmental Microbiology* **65**: 80-87.

Wu Y, He T, Zhong M, Zhang Y, Li E, Huang T & Hu Z (2009) Isolation of marine benzo [a] pyrene-degrading *Ochrobactrum* sp. BAP5 and proteins characterization. *Journal of Environmental Sciences* **21**: 1446-1451.

Yan W, Chi J, Wang Z, Huang W & Zhang G (2009) Spatial and temporal distribution of polycyclic aromatic hydrocarbons (PAHs) in sediments from Daya Bay, South China. *Environmental Pollution* **157**: 1823-1830.

Yergeau E, Arbour M, Brousseau R, Juck D, Lawrence JR, Masson L, Whyte LG & Greer CW (2009) Microarray and real-time PCR analyses of the responses of high-arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. *Applied and Environmental Microbiology* **75**: 6258-6267.

Yousaf S, Ripka K, Reichenauer T, Andria V, Afzal M & Sessitsch A (2010a) Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. *Journal of Applied Microbiology* **109**: 1389-1401.

Yousaf S, Andria V, Reichenauer TG, Smalla K & Sessitsch A (2010b) Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. *Journal of Hazardous Materials* **184**: 523-532.

Yousaf S, Afzal M, Reichenauer TG, Brady CL & Sessitsch A (2011) Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. *Environmental Pollution* **159**: 2675-2683.

Yu S, Ke L, Wong Y & Tam N (2005) Degradation of polycyclic aromatic hydrocarbons by a bacterial consortium enriched from mangrove sediments. *Environment International* **31**: 149-154.

Yunker MB, Macdonald RW, Vingarzan R, Mitchell RH, Goyette D & Sylvestre S (2002) PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. *Organic Geochemistry* **33**: 489-515.

Zachow C, Tilcher R & Berg G (2008) Sugar beet-associated bacterial and fungal communities show a high indigenous antagonistic potential against plant pathogens. *Microbial Ecology* **55**: 119-129.

Zhang W, Song L-s, Ki J-S, Lau C-K, Li X-D & Qian P-Y (2008) Microbial diversity in polluted harbor sediments II: Sulfate-reducing bacterial community assessment using terminal restriction fragment length polymorphism and clone library of *dsrAB* gene. *Estuarine, Coastal and Shelf Science* **76**: 682-691.

Zhang W, Feng H, Chang J, Qu J, Xie H & Yu L (2009) Heavy metal contamination in surface sediments of Yangtze River intertidal zone: an assessment from different indexes. *Environmental Pollution* **157**: 1533-1543.

Zhang W, Wu X, Liu G, Chen T, Zhang G, Dong Z, Yang X & Hu P (2013) Pyrosequencing Reveals Bacterial Diversity in the Rhizosphere of Three *Phragmites australis* Ecotypes. *Geomicrobiology Journal* **30**: 593-599.

Zhong Y, Luan T, Wang X, Lan C & Tam NF (2007) Influence of growth medium on cometabolic degradation of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. strain PheB4. *Applied Microbiology and Biotechnology* **75**: 175-186.

Zhu D, Tanabe S-H, Yang C, Zhang W & Sun J (2013) Bacterial Community Composition of South China Sea Sediments through Pyrosequencing-Based Analysis of 16S rRNA Genes. *PloS one* **8**: e78501.