

M 2015



INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO



FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

**Autochthonous bioaugmentation - a strategy for
enhanced phytoremediation / bioremediation of mixed
contamination in saltmarshes**

INÊS PAES DE FARIA MONTEIRO MONTENEGRO

DISSERTAÇÃO DE MESTRADO APRESENTADA

AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR

DA UNIVERSIDADE DO PORTO EM 30 / 09 / 2015

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Inês Paes de Faria Monteiro Montenegro

**Autochthonous bioaugmentation - a strategy for enhanced
phytoremediation / bioremediation of mixed contamination in
saltmarshes**

Dissertação de Candidatura ao grau de Mestre
em Toxicologia e Contaminação Ambientais
submetida ao Instituto de Ciências Biomédicas
de Abel Salazar da Universidade do Porto

Orientador: Doutora Cristina Marisa Ribeiro de
Almeida, Investigadora Auxiliar no Centro
Interdisciplinar de Investigação Marinha e
Ambiental

Co-orientador: Doutora Ana Paula de Campos
Mucha, Investigadora Auxiliar no Centro
Interdisciplinar de Investigação Marinha e
Ambiental

ACKNOWLEDGMENTS

First of all, I would like to thank my supervisor Doutora Cristina Marisa Ribeiro de Almeida for giving me the opportunity to complete with You my master degree. Thank you for your guidance, encouragement and understanding kindly provided throughout two years.

I would also like to thank my co-supervisor Doutora Ana Paula de Campos Mucha for valuable suggestions and feedback concerning this project.

Special thanks to Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR) and to Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade do Porto, for the use of the installations and facilities. I would like to express my appreciation towards staff (technical/auxiliary) in these institutions.

Thanks for the financial support by the European Regional Development Fund (ERDF) through COMPETE - Operational Competitiveness Program and national funds through FCT, under PEst-C/MAR/LA0015/2013, REEQ/304/QUI/2005, PHYTOBIO (PTDC/MAR/099140/2008), PTDC/AAC-AMB/113973/2009 and ECORISK (reference NORTE-07-0124-FEDER-000054), co-financed by the North Portugal Regional Operational Programme (ON.2 - O Novo Norte), under the National Strategic Reference Framework (NSRF).

ABSTRACT

Large input of nutrients in saltmarshs located in estuaries makes this area one of the most productive ecosystems of the world. On the other hand, in estuaries located in areas highly urbanized and industrialized, there is also a large input of mixed pollutants, coming from rivers and ocean, such as metals/metalloids and organic pollutants. Saltmarsh sediments accumulate these mixtures of pollutants, making these areas a sink of pollutants. Therefore, it is of major importance to restore these areas.

Phytoremediation approaches involving autochthonous plants and associated microorganisms have been explored as a promising alternative for the remediation of these polluted areas. Moreover, the potential of plants for remediation of pollutants can be enhanced by the presence of the microbial communities in their rhizosphere. However, co-contamination of soil/sediments might influence phytoremediation processes, as the different pollutants can interact with each other and with plants and their rhizosphere.

This study aimed firstly to understand the effect of petroleum hydrocarbons on copper remediation potential of *Juncus maritimus* and the role of autochthonous bioaugmentation on this process. In a second step this study aimed to understand the effects of copper on petroleum hydrocarbons remediation potential of *Juncus maritimus* and its rhizospheric microorganisms, as well as, the role of bioaugmentation in this process.

Plants from Lima River estuary (Viana do Castelo; 41° 41' N, 08° 51' W) were collected together with their rhizosediment. In the lab, sediment was separated from plant roots, doped with copper and petroleum and placed in vessels to which plants were transplanted. Vessels were kept in greenhouse, being irrigated through an automated system, regulated to mimic natural tidal cycles that occur in estuarine environments.

For bioaugmentation three types of autochthonous microbial consortia (AMC) were prepared and added to vessels: one resistant to copper, one resistant to petroleum hydrocarbons and another resistant to a mixture of copper and

petroleum hydrocarbons. After five months (experimental time), vessels were dismantled and metal and petroleum hydrocarbons concentrations, as well as the abundance of hydrocarbons degrading microorganisms, were determined in sediments. Metal concentrations were also determined in dried plant organs (roots; rhizomes; stems). Moreover, a sequential extraction procedure was carried out to estimate metal availability in sediments.

Metal concentrations were determined by atomic absorption spectrometry with flame atomization, in dried plant organs and sediments, after digestion with concentrated HNO_3 in a high pressure microwave system. Petroleum hydrocarbon concentrations (estimated through total petroleum hydrocarbon (TPHs) measurements) in dried sediments were analysed by Fourier transform infrared spectrophotometry, after extraction with tetrachloroethylene in a ultrasonic system. The abundance of hydrocarbons degrading microorganisms in sediments was estimated using a modified most probable number (MPN) protocol in 96-well microtiter plates.

Five months after exposure of *J. maritimus* to the sediment doped with copper, metal accumulation in plant roots and rhizomes showed a significant increase. However, in stems, copper concentration remained statistically identical, indicating that no metal translocation occurred. When petroleum was added to the sediment together with copper, and when AMC(Cu) was added to the sediment together with copper plus petroleum, copper levels in *J. maritimus* roots increased significantly, despite the fact that the metal was less available in the sediment. Therefore, the presence of petroleum hydrocarbons and the addition of the microbial consortium resistant to copper improved copper phytostabilization potential of *J. maritimus*, indicating that autochthonous bioaugmentation can have a positive effect.

After the experimental time, 39% hydrocarbons degradation percentage was observed, a percentage that decreased in the presence of copper (to 25%). The addition of microbial consortia resistant to the pollutants (copper and petroleum hydrocarbons, alone or in a mixture) did not improve degradation rates relatively to those observed in the presence of copper without inoculation, indicating that in this case bioaugmentation did not had an effect on the potential hydrocarbon degradation of *J. maritimus* and its associated

rhizospheric microbial community. These results were corroborated by the abundance of hydrocarbon degrading microorganisms, which were not stimulated by the inoculation with the different consortia.

These results should be taken into account when seeking to apply the phytoremediation in the estuaries that may be contaminated with more than one pollutant of different chemical nature.

Resumo

A grande afluência de nutrientes nos sapais salinos localizados em estuários, torna estas áreas num dos ecossistemas mais produtivos do mundo. Por sua vez, nos estuários localizados em áreas densamente urbanizadas e industrializadas, verifica-se também entrada de diversos poluentes, vindos pelos rios e pelos oceanos, tais como metais/metaloídes e poluentes orgânicos. Os sedimentos dos sapais salinos atuam como esponjas absorventes que acumulam estes poluentes, tornando estas áreas verdadeiros receptores/acumuladores de poluentes. Por conseguinte é da maior importância recuperar estas áreas.

A abordagem da fitorremediação que envolve plantas autóctones e respetivos micro-organismos tem sido explorada como uma promissora alternativa para a remediação destas áreas poluídas. Adicionalmente, o potencial das plantas para a remediação de poluentes pode ser aumentado pela presença das comunidades microbianas na sua rizosfera. Porém, a co-contaminação de solos/sedimentos pode influenciar os processos de fitorremediação, isto porque os diferentes poluentes podem interagir entre si, com as plantas e com a sua rizosfera.

Este estudo teve como objetivo, em primeiro lugar investigar o efeito da presença de hidrocarbonetos de petróleo no potencial de remediação da planta de sapal *Juncus maritimus* para sedimentos contaminados com cobre e o papel do bioaumento autóctone sobre este processo de remediação. Uma segunda etapa deste estudo teve como objetivo compreender os efeitos da presença de cobre no potencial de remediação de hidrocarbonetos de petróleo pela planta *Juncus maritimus* em associação com os micro-organismos presentes na sua rizosfera, bem como, num processo de bioaumento.

Plantas do estuário do Rio Lima (Viana do Castelo; 41° 41' N, 08° 51' W) foram recolhidas juntamente com o seu rizossedimento. No laboratório, os sedimentos foram separados, contaminados com cobre e petróleo e colocados em vasos para os quais as plantas foram transplantadas. Os vasos foram mantidos em estufa, sendo irrigados por um sistema automatizado, regulado para simular os ciclos de maré que ocorrem naturalmente em ambientes de sapal estuarino.

Para o bioaumento foram preparados três tipos de consórcio microbiano: um resistente ao cobre, um resistente a hidrocarbonetos de petróleo e outro resistente a uma mistura de hidrocarbonetos de petróleo e de cobre. Após o período experimental (cinco meses), os vasos foram desmanteladas e as concentrações de hidrocarbonetos de petróleo e de metal, bem como a abundância de micro-organismos que degradam hidrocarbonetos, foram determinados nos sedimentos. As concentrações de metal foram também determinadas em diferentes tecidos da planta (raiz, rizoma, caule) previamente secos. Adicionalmente, um procedimento de extração sequencial foi efetuado para estimar a disponibilidade do metal nos sedimentos.

As concentrações de cobre foram determinadas por espectrometria de absorção atômica com atomização por chama, nos tecidos da planta (raiz, rizoma, caule) e no sedimento previamente secos, após digestão com HNO_3 concentrado, em sistema de micro-ondas de alta pressão. As concentrações de hidrocarbonetos de petróleo (estimados por medição de hidrocarbonetos totais de petróleo (TPHs)) nos sedimentos secos foram determinadas por espectrofotometria no infravermelho com Transformada de Fourier, após extração com tetracloretileno num sistema de ultrassom. A abundância de micro-organismos que degradam hidrocarbonetos em sedimentos foi estimada utilizando um protocolo modificado do número mais provável (MPN) em placas de microtitulação de 96 poços.

Cinco meses após a exposição do *J. maritimus* ao sedimento dopado com cobre, a acumulação de metal nas raízes e rizomas apresentou um aumento significativo. No entanto, nos caules a concentração de cobre permaneceu estatisticamente idêntica, indicando que não ocorreu a translocação do metal. A adição de petróleo ao sedimento juntamente com o cobre, e a adição do consórcio microbiano resistente ao cobre (AMC (Cu)), aumentou significativamente os níveis de cobre nas raízes de *J. maritimus*, apesar do facto de o metal se encontrar menos disponível no sedimento. Portanto, a presença de hidrocarbonetos de petróleo e a adição do consórcio microbiano resistente ao cobre (AMC (Cu)), aumentou o potencial de fitoestabilização do cobre pelo *J. maritimus*, indicando que bioaumento autóctone pode ter tido um efeito positivo.

Após o período experimental, foi observada uma percentagem de

degradação de hidrocarbonetos de 39% (estimado por análise de TPHs) uma percentagem que diminuiu na presença de cobre (25%). A adição de consórcios microbianos resistentes aos poluentes (hidrocarbonetos de petróleo e/ou de cobre, isolado ou em conjunto) não melhorou a taxa de degradação relativamente aos observados na presença de cobre sem inoculação, indicando que, neste caso o bioaumento não teve um efeito sobre o potencial degradador de hidrocarbonetos do *J. maritimus* e da comunidade microbiana associada presente na rizosfera. Estes resultados foram corroborados pela abundância de microrganismos degradadores de hidrocarbonetos (estimada por MPN), que indicou não ter sido estimulada pela inoculação com os diferentes consórcios.

Estes resultados devem ser tidos em conta quando se pretende aplicar a fitorremediação nos estuários que podem estar contaminados com mais do que um poluente e de natureza química diferente.

(Texto efetuado ao abrigo do novo acordo ortográfico)

CONTENTS

Acknowledgments	I
Abstract	III
Resumo	VII
Contents	XI
Figure Index	XV
Table Index	XVII
List of abbreviations	XIX
Chapter I – Introduction	1
1. Estuarine saltmarshes	3
1.1. Saltmarsh plants.....	4
1.2. <i>Juncus maritimus</i>	4
2. Phytoremediation	6
2.1. Technology description.....	8
2.2. Microorganisms-assisted phytoremediation.....	9
3. Inorganic pollutants (metals/metalloids)	11
3.1. Copper.....	13
3.2. Phytoremediation of inorganic pollutant.....	15
3.3. Saltmarshes and phytoremediation of metals/metalloids.....	20
4. Organic pollutants	21
4.1. Petroleum.....	22
4.2. Phytoremediation of organic pollutants.....	24
4.3. Saltmarshes and phytoremediation of organic pollutants.....	27
5. Co-contamination	27
5.1. Impact of organic pollutants in metals/metalloids phytoremediation.....	28

5.2. Impact of metals on biodegradation of organic contaminants.....	29
5.3. Bioremediation in co-contaminated environments.....	31
6. Aims of the work.....	32
 Chapter II – Material and methods.....	 35
1. Sampling.....	37
2. Sediment spiking.....	37
3. Microbial consortia preparation and inoculation.....	39
4. Microcosms ' experiments.....	40
5. Analytical determinations.....	42
5.1. Material preparation.....	42
5.2. Sample processing.....	42
5.3. Metal determinations.....	43
5.4. Metal availability in sediments.....	43
5.5. Total petroleum hydrocarbons (TPH) determinations.....	44
5.6. Hydrocarbon degrading microorganisms abundance.....	45
6. Data analysis.....	45
 Chapter III - Effect of petroleum hydrocarbons in copper phytoremediation by a saltmarsh plant (<i>Juncus maritimus</i>) and the role of autochthonous bioaugmentation.....	 47
1. Results.....	49
1.1. Plant biomass.....	49
1.2. Metal levels.....	52
1.2.1. Metal levels in plant organs.....	52
1.2.2. Metal levels in sediment.....	57
1.3. Translocation Factor (TF) and Enrichments Factors (EF).....	57

1.4. Metal fractionation.....	59
2. Discussion.....	61
2.1. Plant biomass.....	61
2.1.1. Influence of pollutants.....	61
2.1.2. Influence of autochthonous microbial consortium.....	63
2.2. Metal levels.....	64
2.2.1. Influence of pollutants.....	66
2.2.2. Influence of autochthonous microbial consortium.....	69
3 Conclusions.....	73
 Chapter IV – Effect of copper in petroleum hydrocarbons biodegradation by a saltmarsh plant (<i>Juncus maritimus</i>) - rhizosphere microorganisms association and the role of autochthonous bioaugmentation.....	 75
1. Results.....	77
1.1. Plant biomass.....	77
1.2.Total Petroleum Hydrocarbons (TPH) in sediment.....	80
1.3.Abundance of hydrocarbons degrading microorganisms.....	82
2. Discussion.....	85
2.1.Plant biomass and contaminants.....	86
2.2.Plant biomass and bioaugmentation.....	87
2.3 Effect of copper on petroleum bioremediation.....	88
2.4.Effect of bioaugmentation on petroleum biodegradation.....	91
3. Conclusions	93
 Chapter V – Conclusions and future directions.....	 95
 Bibliography.....	 101

FIGURES INDEX

Introduction		Page
Figure 1	<i>J. maritimus</i> plants on their natural habitat. Picture retrieved from Msc Thesis of Nunes da Silva, 2012).	5
Figure 2	Illustration of anatomical details of the <i>Juncus maritimus</i> . Assembly made by Inês P.F.M. Montenegro.	5
Figure 3	Plant-associated microorganisms of rhizosphere in the remediation process in metals/metalloids contaminated soils by raising metal mobilization/immobilization. “(a) plant-associated microbes improve plant metals/metalloids uptake by producing metal mobilizing chelators. Plant associated microbes can may reduce plant metals/metalloids uptake and/or translocation through (b) producing metal immobilizing metabolites, (c) metals/metalloids reduction and/or (d) metals/metalloids biosorption. Abbreviations: extracellular polymeric substances (EPS)” (modified image by Inês PFM Montenegro from Rajkumar <i>et al.</i> (2012)).	19
Figure 4	Schematic representation of the enzymatic and microbial activities for remediation of organic pollutants in rhizosphere (modified image by Inês PFM Montenegro from Abhilash <i>et al.</i> (2009)).	25
Materials and methods		
Figure 5	(A) Picture of the sampling of <i>J. maritimus</i> in Lima River estuary (Picture retrieved from: Msc Thesis of Nunes da Silva, 2012). (B) Illustration of the sampling, plant with sediment attached to its roots (cubes with 20 ³ cm ³) (assembly made by Inês PFM Montenegro).	37
Figure 6	Flow diagram of the procedure used for sediment spiking. Assembly made by Inês PFM Montenegro.	38
Figure 7	Flow diagram of the procedure used to produce an autochthonous microbial consortium (AMC). Assembly made by Inês PFM Montenegro.	39
Figure 8	(A) Picture of the vessels with a layer of pebbles at the bottom; (Picture retrieved from Msc Thesis of Nunes da Silva, 2012). (B) Illustration of details in plastic vessels, layer of pebbles and draining tap at the bottom. Assembly made by Inês PFM Montenegro.	40
Figure 9	Flow diagram of the procedure used for the assemble experiment. Assembly made by Inês PFM Montenegro.	41
Figure 10	Greenhouses where plants were kept during five months with an irrigation solution through an automated irrigation system regulated to mimic the tidal cycles natural (six hours of drought and six hours of flood.) (image retrieved from Msc Thesis of Nunes da Silva, 2012).	42
Results		
Figure 11	Percentage (%) of each plant organ weight relatively to the total biomass of <i>J. maritimus</i> in the several treatments.	49

Figure 12	Total copper concentration ($\mu\text{g/g}_{\text{plant organ}}$) in stem, rhizomes and roots of <i>J. maritimus</i> , five months after doping (mean and standard deviation, $n=3$) each treatment (doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)). Control treatment had no doping. The values represent the means of three replicates $\pm$ standard deviation. Columns showing different letters, the differences are statistically significant ($p<0.05$, <i>t</i> -student) and with the same letter the differences are not statistically significantly ($p>0.05$, <i>t</i> -student).	51
Figure 13	Total copper concentration in sediment after five months (mean and standard deviation, $n=3$), for each treatment (doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)). Columns showing different letters, the differences are statistically significant ($p<0.05$, <i>t</i> -student) and with the same letter the differences are not statistically significantly ($p>0.05$, <i>t</i> -student).	55
Figure 14	Percentages (%) of each metal fraction relative to the total metal concentrations present in the rhizosediment of each treatment.	58
Figure 15	Percentage (%) of each plant organ weight (roots; rhizomes; leaves and stems) relatively to the total biomass of <i>J. maritimus</i> in the several treatments.	78
Figure 16	TPHs concentration in sediment ($\text{mg/g}_{\text{sediment}}$) at initial time (T0) and in different treatments after five month (T5). Means followed by different letters are significantly different ($p<0.05$, <i>t</i> -student) and with the same letters indicate differences not statistically significantly ($p>0.05$, <i>t</i> -student). Error bar show standard deviation ($n=3$).	79
Figure 17	The number of hydrocarbons degraders microorganisms (average (Log MPN/ $\text{g}_{\text{dry sediment}}$) and standard deviation, $n=3$) in several treatment (control; doped with Cu (Cu); doped with petroleum (Pet); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu+Pet)). Values are showing different letters indicate significant differences ($p<0.05$, <i>t</i> -student) and the same letters indicate differences not statistically significantly ($p>0.05$, <i>t</i> -student).	82

TABLES INDEX

Results		Page
Table 1	Dry weight (g) of the plant organs (average and standard deviation, n=3) for each treatment (control; doped with petroleum (Pet); doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)). Plant total weight per treatment is also included. Values showing different letters indicate significant differences ($p < 0.05$, t -student) and with the same letters indicate differences not statistically significantly ($p > 0.05$, t -student).	48
Table 2	Correlation of the average biomass between several organs of the <i>J. maritimus</i> .	48
Table 3	Correlation between treatments in copper concentration present in each organ. The (=) sign is an indication of not significantly different ($p > 0.05$) in copper concentrations between treatment (#) and (##). The (+) sign is an indication of increasing the (-) sign indicates decrease.	51
Table 4	Correlation of the average of the copper concentration ($\mu\text{g/g}_{\text{plant organ}}$) between several organs of the <i>J. maritimus</i> .	53
Table 5	Translocation Factor (TF)* and Enrichments Factors (EF)**.	56
Table 6	Metal fractionation in rhizosediment (average ($\mu\text{g/g}_{\text{sediment}}$) and standard deviation, n=3) for each treatment (doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)). Cu concentrations in the different fractions are presented in $\mu\text{g/g}_{\text{sediment}}$. Values showing different letters, indicate significant differences ($p < 0.05$, t -student) and with the same letter the differences are not statistically significantly ($p > 0.05$, t -student).	57
Table 7	Dry weight (g) of the plant organs, (average and standard deviation, n=3) for each treatment (control; doped with petroleum (Pet); doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu); doped with Cu plus petroleum and AMC resistant to Pet (Cu+Pet+AMC(Pet); doped with Cu plus petroleum and AMC resistant to Cu plus petroleum (Cu+Pet+AMC(Cu+Pet)). Plant total weight per treatment is also included. Values showing different letters indicate significant differences in each plant organ ($p > 0.05$, t -student).	76
Table 8	Correlation of the average biomass between several organs of the <i>J. maritimus</i> .	77
Table 9	TPHs degradation rates (%) in sediment five months (T5) after contamination of the sediment, in the different treatments: doped with petroleum (Pet); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu); doped with Cu plus petroleum and AMC resistant to petroleum (Cu+Pet+AMC(Pet); doped with Cu plus petroleum and AMC resistant to Cu plus petroleum (Cu+Pet+AMC(Cu+Pet)).	80
Table 10	Correlation between treatments in the numbers of the HD microorganisms present in sediment and calculated by MPNs protocol. The (=) sign is an indication of not significantly different ($p > 0.05$) between treatments (#) and (##) in the numbers of the HD microorganisms present in sediment. The (+) sign is an indication of increasing the (-) sign indicates to decrease.	83

LIST OF ABBREVIATIONS

AMC - Autochthonous Microbial Consortia

AMC(Cu) - Autochthonous Microbial Consortium resistant to copper

AMC(Pet) - Autochthonous Microbial Consortium resistant to petroleum hydrocarbons

AMC(Cu+Pet) - Autochthonous Microbial Consortium resistant to copper plus petroleum hydrocarbons

Ag- Silver

As – Arsenic

Al - Aluminium

ATP – Adenosine triphosphate

BH – Bushnell Haas

Ca - Calcium

Cd- Cadmium

Co - Cobalt

CO₂ – Carbon dioxide

Cr – Chromium

Cs - Cesium

Cu- Copper

C₂Cl₄- Tetrachloroethylene

EF - Enrichments Factor

ERM – Effect Range Median

Fe - Iron

HD - Hydrocarbon degrading microorganisms

HNO₃ - Nitric acid

H₂O₂ - Hydrogen peroxide

IUPAC – International Union of Pure and Applied Chemistry

J. maritimus - *Juncus maritimus*

K - Potassium

MAHs- Monocyclic Aromatic Hydrocarbons

Mg - Magnesium

Mn – Manganese

Mo - Molybdenum

MPN – Most Probable Number

Na – Sodium

Na₂SO₄ - Anhydrous sodium sulfate

P. australis - *Phragmites australis*

P - Phosphorus

PAHs - Polycyclic Aromatic Hydrocarbons

PCP - pentachlorophenol

Pet - Petroleum

PTFE - Polytetrafluoroethylene

ROS - Reactive oxygen species

Sr - Strontium

TF - Translocation Factor

TPH - Total Petroleum Hydrocarbons

T. striata - *Triglochin striata*

U - Uranium

T0 - Initial time (time zero days)

T5 - Five month after initial time (time one hundred fifty days)

Zn- Zinc

CHAPTER I

Introduction

1. Estuarine saltmarshes

1.1. Saltmarsh plants

1.2. *Juncus maritimus*

2. Phytoremediation

2.1. Technology description

2.2. Microorganisms-assisted
phytoremediation

3. Inorganic pollutants (metals/metalloids)

3.1. Copper

3.2. Phytoremediation of inorganic
pollutant

3.3. Saltmarshes and phytoremediation
of metals/metalloids

4. Organic pollutants

4.1. Petroleum

4.2. Phytoremediation of organic
pollutants

4.3. Saltmarshes and phytoremediation
of organic pollutants

5. Co-contamination

5.1. Impact of organic pollutants in
metals/metalloids phytoremediation

5.2. Impact of metals on biodegradation
of organic contaminants

5.3. Bioremediation in co-contaminated
environments

6. Aims of the work

Introduction

1. Estuarine saltmarshes

Estuarine areas represent a dynamic system, shaped by the interaction of water, sediment and vegetation and are important ecosystems for an endless number of species that comes from contiguous terrestrial/aquatic environments (Lefeuvre *et al.*, 2003). A very wide range of species of invertebrates and vertebrates depend on these habitats as places to live (including in the earliest stages of life), feed and reproduce, being these sites also part of the routes of migratory species. Not only these sites are important because they provide a habitat for many species of organisms, including many commercially important species, and have high rates of primary production (Vernberg, 1993), they also provide important *ecosystem services* such as water purification and habitat protection (Barbier *et al.*, 2011).

Location usually close to urban areas with intense anthropogenic activity, that receives inputs from hydrographic basins, makes estuarine sediments to act as sinks of variable pollutants, either inorganic pollutants, such as metals (e.g. copper) and metalloids (e.g. arsenic) (Nieto *et al.*, 2007; Deycard *et al.*, 2014), or organic pollutants, such as petroleum hydrocarbons (Ribeiro *et al.*, 2014). These pollutants may be traced to a particular point source, such as urban/industrial sewage or accidental spills. For instance, *Prestige* oil spill in the Galician coast in Spain 2002 was a large environment disaster with a high impact on the coastal area as described by Kirby and Law (2010). Pollutants may also result from a large area, from non-point sources, such as runoff after rain events, human activities along coastlines, and navigation activities.

Restore estuarine saltmarsh areas, with minimum impact in their ecosystem functions and services, becomes a mandatory requirement. An option for restoration of the estuarine saltmarsh areas, involves the application of phytoremediation technologies.

1.1. Saltmarsh plants

Saltmarshes are covered with salt-tolerant plant species - halophytes plants (native and invasive species), plants capable of completing their life cycle in salt concentration around 0.2 M NaCl or even higher and in an area subject to recurrent flooding (Oliveira *et al.*, 2015). Halophytes have been referred to as naturally better adapted to cope with environmental stresses (Manousaki and Kalogerakis, 2011).

Several species of saltmarsh have studies in context of remediation of metals/metalloids or/and organic pollutants, for instance: *Halimione portulacoides* (e.g. Almeida *et al.*, 2008; Machado *et al.*, 2012); *Spartina maritima* (e.g. Reborela and Caçador, 2007); *Phragmites australis* (e.g. Ribeiro *et al.*, 2011; Oliveira *et al.*, 2014); *Juncus maritimus* (e.g. Almeida *et al.*, 2004; Nunes da Silva *et al.* 2014; Ribeiro *et al.*, 2014); *Scirpus maritimus* (e.g. Almeida *et al.*, 2006a; Mucha *et al.*, 2008); *Triglochin maritimus* (e.g. Castro *et al.*, 2009); *Triglochin striata* (e.g. Ribeiro *et al.*, 2011).

Through these studies it appears that saltmarsh plants may play an important role in removing pollutants (inorganic/organic) from estuarine ecosystems, for instance, directly by phytoremediation (e.g. accumulation of metals in plant; Almeida *et al.*, 2008) or by capability to retain pollutants around the roots (e.g. retain hydrocarbons; Ribeiro *et al.*, 2011) (e.g. phytostabilization of metals; Almeida *et al.*, 2006a). Indirectly by release low molecular weight organic acids (e.g. Mucha *et al.*, 2005) and release of strong organic ligands (in hydroponics situation; e.g. Mucha *et al.*, 2008), with capacity for complex with metals and controlling metal bioavailability and/or speciation, or by influence in the microbial community, through fostering the development of hydrocarbon-degrading microbial populations in its rhizosphere (e.g. Ribeiro *et al.*, 2011).

1.2. *Juncus maritimus*

Plants (*J. maritimus*) and sediments (rhizosediments) for this study were collected in Lima River estuary. Contemplating saltmarshes vegetation communities, the Lima River estuary (NW Portugal) is part of the Eurosiberian regions of Portuguese coastline (Costa *et al.*, 2009), with an abundant salt

meadow where is commonly found halophytic communities, composed by *Juncus maritimus*, *Triglochin striata* (non-native), *Phragmites australis* (invasive) and *Spartina patens* (Almeida *et al.*, 2011).

J. maritimus (Fig. 1) is a native species in Atlantis coast of Europe and particularly in Portuguese saltmarshes (Ribeiro *et al.*, 2013a). This plant is angiosperms-monocotyledon and perennial, from Juncaceae family. Is differentiated into a subterranean long-lived horizontal modified stem (rhizome), lateral shoots and roots adventitious from rhizome, leafy short shoots and an above-ground ephemeral flowering stem (Balslev, 1998), as show in Fig. 2.



Figure 1 *J. maritimus* plants growing on their natural habitat. (Picture retrieved from Msc Thesis of Nunes da Silva, 2012).

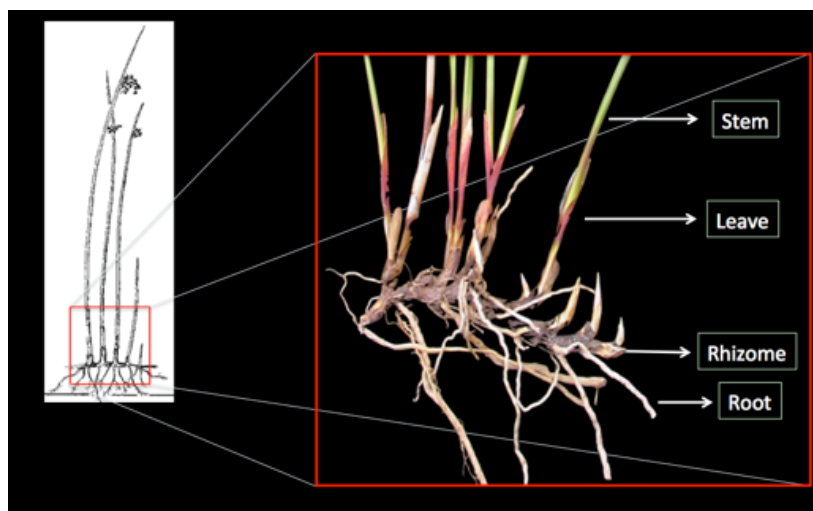


Figure 2 Schematic representation of anatomical details of the *J. maritimus*. Assembly made by Inês PFM Montenegro.

This plant grows on the intertidal zone in saltmarsh and has the endogenous biochemical capacity to influence the composition of rhizosediments, for instance, Almeida et al. (2004) reported “*J. maritimus* markedly influenced the sediments among its roots and rhizomes, changing metal distribution and speciation”. *J. maritimus* change the available of the metal for uptake by releasing organic acids (e.g. Mucha *et al.*, 2005), change pH and redox potential (Figueira *et al.*, 2012). In case of the organic pollutants, *J. maritimus* and their associated microorganisms present a potential for remediation of petroleum hydrocarbons (Ribeiro *et al.*, 2014). In the case of metal, *J. maritimus* was shown to have potential for phytoextraction (or phytostabilization) Cd, Cu, and Zn (e.g. Almeida *et al.*, 2004).

2. Phytoremediation

Phytoremediation derived from a combination of the Greek word *phytos* (plant) and the Latin word *remediation* (roughly translated as restoration of balance/equilibrium) (Barbafieri *et al.*, 2013).

Phytoremediation is a generic term that covers different processes applying vascular plants and associated microorganisms to environmental cleanup, i.e., *in situ* treatment of contaminated sediments, soils and water globally and applied to both inorganic and organic pollutants (Nwoko, 2010; Barbafieri *et al.*, 2013).

The concept of using green plants to clean up contaminated environments is not new. About three hundred years ago plants have been proposed for use in the treatment of wastewater and at end of the 19th century, the first plant species was documented by accumulating high levels of metals in leaves (Lasat, 2000).

The traditional term of phytoremediation has been recently supplanted by the term “phytotechnology”, used to indicate “the application of science and engineering to study problems and provide solutions involving plants” (UNEP, 2003), this includes other methods beyond remediation such as constructed

wetlands or ground cover plants for minimizing erosion (Pilon-Smits, 2005; Reichenauer and Germida, 2008).

Phytoremediation is an environmental biotechnology that has captivated the interest of scientists, public opinion, regulators and public administration (Marmiroli *et al.*, 2006). Phytoremediation is deemed to date, the only solution that approaches the problem of remediation of environmental pollution, from an eco-sustainable point of view, i.e., environmentally friendly and relatively economic (Barbafieri *et al.*, 2013). In fact, phytoremediation is an environmental friendly technology that protects the ecosystem stability (noninvasive), causing minimum damage (Shilev *et al.*, 2009; Rani and Juwarkar, 2013), with low implementation cost and publicly acceptable way to address the removal of environmental contaminants (Arthur *et al.*, 2005).

Several organizations have given enface to the application of this technology. For example, the United Nations Environment Programme (UNEP) recognizes as important sustainable development and promotes the incorporation of phytotechnologies as part of a solution for environmental pollution.

On that basis, the European Commission proposed within the Directive 2008/1/EC, a guideline to select the most suitable technique according to criteria such as environmental friendliness, preexisting scientific knowledge, or required time (Barbafieri *et al.*, 2013). These guidelines allow the involved to choose the best remediation technology for a contaminated area, considering the economic, environmental, and social variables (Conesa *et al.*, 2012).

Phytoremediation is especially important for removing metals/metalloids and organic pollutants from water, soil and sediments (Huesemann, 2004; Gerherdt *et al.*, 2009; Dordio and Carvalho, 2013; De Lange *et al.*, 2013). However phytoremediation has limitations, for example, it is limited to soil properties, low level of contamination, confined to rooting depth, and to fraction bioavailable (Pilon-Smiths, 2005), and the remediation process could affect the food chain when chemical are degraded and uptake by plant. The treatment time, typically several years, is seen as the biggest impediment to have commercial significance (Kirpichtchikova *et al.*, 2006) and efforts have to be made to increase the pace of the biological processes involved.

On the other hand, non-biological remediation technologies and bio/phytoremediation are not mutually exclusive, because pollution distribution and concentration are not homogeneous, and more efficient, and cost-effective remediation solutions will be the implementation of the combination of different technologies (Pilon-Smiths, 2005).

2.1. Technology description

The application of phytoremediation technologies is based on the type and speciation of the pollutant, and on characteristics of the plant species (Salt *et al.*, 1998).

Phytoextraction (Phytoaccumulation) is the uptake/absorption and translocation of contaminants (metal or organic) by plant roots into the aboveground structures of the plants, that can be harvested and burn, gaining energy and, for instance, recycling the metal from the ash (Salt *et al.*, 1998; Tangahu *et al.*, 2011).

Phytostabilization: its application aims at the use of plants to reduce the bioavailability of soil pollutants on adjacent environmental compartments through adsorption and accumulation in plant roots, or precipitation within the root zone, and is most effective on soil with high levels of contamination of metals and metalloids, other organic pollutants, and crude oil residues (Tangahu *et al.*, 2011; Barbafieri *et al.*, 2013).

Phytodegradation (or Rhyzodegradation): its application aims at the use of plants and activity of the microorganisms of the rhizosphere to degrade organic pollutants (Alkorta *et al.*, 2004).

Rhizofiltration is the ability of the roots or root exudates to create conditions that lead to in precipitation of the contaminant. The contaminant may remain associated with the roots (adsorption) or may be taken up (absorption) and

retained in the roots or translocated to other parts of the plant, depending on the plant species, type of contaminant and its concentration in solution surrounding the root zone (Tangahu *et al.*, 2011; Rani and Juwarkar, 2013).

Phytovolatilization is the uptake and translocation of a contaminant in a plant and release of the volatile form (contaminant or a modified form thereof) to the atmosphere (Pilon-Smits, 2005, Tangabu *et al.*, 2011).

Recently new strategies for the process of remediation have been uncovered, through the application of nanoparticles, non-living biomass and genetically modified plants (Gaur *et al.*, 2014).

2.2. Microorganisms–assisted phytoremediation

Phyto and bio-remediation technologies cannot be considered independent of each other, because the microorganisms play an important role in phytotechnologies (Reichenauer and Germida, 2008). Since microorganisms from the rhizosphere have a greatly influence in the growth and survival of plants in contaminated sites, phytoremediation methods that exploit rhizosphere bacteria have received considerable attention (Glick 2010; Ma *et al.*, 2011).

Bioremediation may be defined as the use of living organisms to remove environmental pollutants. This process can occur naturally, the so called bioattenuation. But it can also be enhanced by two methods, *bioaugmentation* or *biostimulation* (Sarkar *et al.*, 2005).

In fact, there are three different types *in situ* of bioremediation process: (i) *bioattenuation* (natural process of degradation) (ii) *bioaugmentation* (addition of pre-grown microbial culture resistant to the pollutant to enhance its degradation, (iii) *biostimulation* (injection of nutrients and other supplementary components to induce propagation of the native microbial population) (Megharaj *et al.*, 2011; Tyagi *et al.*, 2011).

Bioattenuation or natural attenuation processes is the transformation (biodegradation) of the pollutants into less harmful forms or their immobilization, by microorganisms, and to some extent by the reactions with

naturally –occurring chemicals and sorption on the geologic media (Megharaj *et al.*, 2011). The time required for this processes varies largely with site conditions and is contaminant-specific. For instance, it is accepted as a method for treating fuel components but not for many other classes of the contaminant (Megharaj *et al.*, 2011).

Bioaugmentation is the application of bacteria pre-adapted to the contaminant to the target environment. The “soil activation” is a concept which is based on the cultivation of biomass from a fraction of a contaminated soil and the subsequent use as an inoculum for bioaugmentation, for the same contaminated soil. For instance, the soil with microbiota, adapted by prior exposure to petroleum hydrocarbon can be a source of microorganisms for remediating soils freshly contaminated with this pollutant (Megharaj *et al.*, 2011). This process, pre-exposure and subsequent re-exposure to a pollutant can contribute to improve survival, persistence and degradability activities of microorganisms, leading to enhanced remediation of the polluted soil (Megharaj *et al.*, 2011).

Bioaugmentation has been utilized in agriculture for many years (Gentry *et al.*, 2004), but more recently it has been applied as a strategy to remediate environmentally polluted sites (e.g. saltmarsh sediments; Teixeira *et al.*, 2014). Bioaugmentation has been shown to increase degradation of numerous compounds including chlorinated solvents, pentachlorophenol, PAHs, pesticides and oil. However, some studies have demonstrated that bioaugmentation, often does not lead to increased contaminant remediation (Gentry *et al.*, 2004).

Bioaugmentation can be realized in three ways: (i) the enrichment or isolation of indigenous microorganisms from the polluted site, their subsequent culturing and re-inoculation into the sites to be remediated, (ii) inoculation into the sites to be remediated with microorganisms with known degradation potential for the pollutant in question, but which are exogenous to the polluted site, (iii) inoculation of constructed or force-mutated microorganisms (Zawierucha and Malina, 2011).

Bioaugmentation that use indigenous microorganism to the sites to be remediated is defined as *autochthonous bioaugmentation*. The use of indigenous microorganisms with adapted biochemical potential was proved to be one of the most powerful tools for bioaugmentation (Zawierucha and Malina, 2011).

When exogenous microbial populations are introduced into contaminated soils sometimes a considerable number of deaths occur. There are several explanations for these deaths, which include both abiotic and biotic stress factors (Gentry *et al.*, 2004). The abiotic factors include fluctuations or extremes in temperature, water content, pH, and nutrient availability, and potentially toxic pollutant levels in contaminated soil, and the biotic factors include the competition from indigenous organisms for limited nutrients, along with antagonistic interactions including antibiotic production by competing organisms and predation interactions by protozoa and bacteriophages (Gentry *et al.*, 2004). Indigenous microorganisms present in contaminated sites is surely adapted to the climatic, physicochemical and nutrient conditions (abiotic factors) (Zawierucha and Malina, 2011).

Biostimulation of the microbiological processes for contaminant degradation usually involves the modification of the contaminated medium by adjusting pH, improving the soil moisture, append microbial products such as (bio)surfactants or enzymes, (Singh *et al.*, 2011) and administering nutrients as organic and/or inorganic fertilizers (addition of limiting nutrients to achieve an ideal nutrient C:N:P) into a contaminated system. This process aims to promote an increase of the population of indigenous microorganisms, including of those able to degrade the pollutant (Sarkar *et al.*, 2005). The process of biostimulation introduces additional nutrients which increase the population of the indigenous microorganisms. These microorganisms may or may not primarily target the contaminant as a food source, however the degradation is in general, more quicker in comparison to natural attenuation due to the increased population (Zawierucha and Malina, 2011).

3. Inorganic pollutants (metals/metalloids)

The term *heavy metals*, generally refers in various publications (e.g. White, 2012), as a group of metals and semimetals (metalloids) that have been associated with contamination with highly toxic or ecotoxic properties (Duffus, 2002). However, the same author considers “the term *heavy metal* has been used inconsistently”, and “to avoid the use of the term *heavy metal*, a new

classification based on the periodic table is needed”, this “leads to confusion regarding the significance of the term”. The inexact use of the term, without no authoritative definition, sometimes results in list of *heavy metals* differ from one set of regulations of another and the term is sometimes used without even specifying which *heavy metals* are covered (Duffus, 2002).

Inorganic pollutants (metals/metalloids) emerge as natural elements in the Earth's crust or atmosphere, (Pilon-Smith, 2005) at levels that are regarded as trace (< 1000 mg/kg) and rarely toxic. However, metals become a contaminant in the soil environments because “ (i) their rates of generation via manmade cycle are more rapid relative to natural ones, (ii) they become transferred from mines to random environmental location where higher toxicity potentials of direct exposure occur, (iii) the concentration of the metals in discarded products are relatively high compared to those in the receiving environment, and (iv) the chemical form (species) in which a metal is found in the receiving ecosystem may render is more bioavailable” (Wuana and Okieimen, 2011).

Metals become considered as a “contaminant” if it occurs where it is undesirable (Panagos *et al.*, 2013) or at certain concentrations. But when “contaminant” causes adverse human or environmental effect they are considered “pollutant” (Chapman, 2007). “All pollutants are contaminants, but not all contaminants are pollutants” because “a substance introduced into the environment may be almost bioavailable to organisms depending on their chemical form, modifying factors in the environment, the environmental compartment they occupy, and the reactions (behavioural and physiological) of exposed biota” (Chapman, 2007).

Several technologies to cleanup soil contaminated with metals/metalloids (*in situ* and *ex situ*) have been employed, the most common is incineration, disposal in landfill, flotation, electroremediation, bioleaching, phytoremediation and soil washing with chemicals (Kirpichtchikova *et al.*, 2006).

The most common contaminants metals/metalloids are lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), copper (Cu), nickel (Ni), silver (Ag), zinc (Zn), arsenic (As), and the less common metallic contaminants include aluminum (Al),

cesium (Cs), cobalt (Co), manganese (Mn), molybdenum (Mo), strontium (Sr), and uranium (U) (Panagos *et al.*, 2013).

Several of these elements, as is the case of Cu, are essential for plants growth and development, since they are constituents of many enzymes and other proteins, which cannot be replaced by another element due to unique physiological or biochemical performing (White, 2012). However, elevated concentrations can lead to toxicity symptoms and growth inhibition of plants (Yadav, 2010).

3.1. Copper

Copper in soil

Copper is a transition metal, which belongs to the period 4 and group 11 (by modern IUPAC numbering) of the periodic table. With chemical symbol Cu, atomic number 29, atomic weight 63.5, occur in oxidation stage (+1) and (+2), and it has a density of the 8.92g/cm³, melting point 1083°C and boiling point 2595°C (Kabata-Pendias, 2011; Wuana and Okieimen, 2011).

Copper occurs in the Earth's crust at a concentration average of 55mg/Kg, which makes copper the element with greater relatively terrestrial abundance compared to other metals of its group (silver and gold) (Kabata-Pendias, 2011). Copper displays a strong affinity for sulfur, forming different complexes, such as chalcopryite (CuFeS₂), bornite (Cu₅FeS₄), chalcocite (Cu₂S) and covellite (CuS), being often associated with sphalerite (ZnS), pyrite (FeS), galena (PbS) and its ores that are found in acid igneous rocks and in sedimentary deposits (Kabata-Pendias, 2011). During the physical and chemical weathering of copper sulfides, copper is introduced into oxide and carbonate minerals (cuprite (Cu₂O), tenarite (CuO), malachite (Cu₂CO₃(OH)₂), azurite (Cu₂(CO₃)₂(OH)₂)) (Kabata-Pendias, 2011).

Generally this metal exhibits little variation in its total content in soil profiles, being a rather immobile element in soil. Copper appears accumulated in the upper few centimeters of soils, however, because of its tendency to be adsorbed by soil organic matter, carbonates, clays minerals and oxyhydroxides of manganese (Mn) and iron (Fe), it may be also accumulated in deeper soil layers

(Kabata-Pendias, 2011).

However, reactions of copper with active groups at the surface of the solid phase, reaction of copper with specific substances and release by desorption process of the copper in soil contribute to the presence of this metal in soil solutions (Kabata-Pendias, 2011).

The most common forms of copper in soil solutions are those associated with organic chelates. The bioavailability of soluble forms of copper is associated probably with the molecular weight of the copper complexes and with the amounts present. But copper toxicity to plants, bioavailability will be influenced by species of copper and, not just a function of its total concentration (Kabata-Pendias, 2011).

It is axiomatic that the solubility and bioavailability of copper are dominated by various biogeochemical soil factors: pH, temperature, redox potential, soil organic matter, soil texture, mineral composition, and water regime (Kabata-Pendias, 2011).

Copper in plants

As mentioned, copper is an essential metal for the life of plants, is a constituent of several “key” enzymes like plastocyanin and cytochrome oxidase, i.e., plays an important role in physiological processes like photosynthetic system and respiratory electron transport chain (ATP synthesis and CO₂ assimilation) (Yadav 2010), but also in the carbohydrate and nitrate metabolisms; water permeability; reproduction; disease resistance (Kabata-Pendias, 2011). It is classified as a micronutrient because only a small amount is needed, however, the deficiency strongly affects physiological processes and therefore plant development (Kabata-Pendias, 2011). Excess of copper in soil leads to plant growth retardation and leaf chlorosis, induces oxidative stress and reactive oxygen species (ROS) causing disturbance of metabolic pathways and damage to macromolecules and consequently damage in cell (Yadav, 2010).

3.2. Phytoremediation of inorganic pollutants

Unlike many organic contaminants that are oxidized to carbon (IV) oxide by microbial action, metals do not suffer degradation. In fact, the only possible changes that metals may suffer are in their chemical forms (speciation) and bioavailability (Wuana and Okieimen, 2011). Therefore, metals can persist in soils for a long time, accumulating in the environment and subsequently contaminating the food chain, which may represent a risk to environmental and human health (Wuana and Okieimen, 2011; Ali *et al.*, 2013).

Phytoremediation can be an option to reduce the metal loads of metal contaminated soil or waters.

Phytoremediation of inorganic pollutants may have two paths: (1) reduction of the metal mobility through absorption/adsorption, or precipitation by plant roots, hence, decreasing their bioavailability (phytostabilization); (2) uptake by plant roots and translocation to aboveground parts of the plant (phytoextraction) (Mendez and Maier, 2008; Peng *et al.*, 2008).

For inorganic pollutants like mercury (Hg) and arsenic (As), some plants can uptake these elements by roots, transfer to the aboveground structures and by transpiration process release them into the atmosphere through the leaf stomata, the so-called phytovolatilization (Sheoran *et al.*, 2011).

But phytoremediation potential is highly dependent on metal bioavailability and on plant's capacity to uptake and tolerate metals.

Bioavailability of metals and metalloids in soil

In soil, metals/metalloids exist in bioavailable and non-bioavailable form (Tak *et al.*, 2013). The bioavailability of metals/metalloids in soil is a critical factor affecting the efficiency of their extraction by plants (Ali *et al.*, 2013). Because of the high binding tendency that metallic micronutrient have for soil particles, plants have developed several strategies to increase metals bioavailability in soil, as a response to their deficiency (Lone *et al.*, 2008). These strategies include secretion of metal-chelating compounds by plant roots into the rhizosphere, for instance the so-called phytosiderophores (Lone *et al.*, 2008). Metal ions chelated to phytosiderophores can be transported across the plasma

membrane as a metal-phytosiderophore complex via specialized transporters (Salt *et al.*, 1998). Another strategy is secretion of organic acids by plants roots, such as malic, malonic, or oxalic acids (Mucha *et al.*, 2005), which act as chelators and decrease the rhizosphere pH (e.g. Sundby *et al.*, 2005) thus making metal cations more bioavailable. This can facilitate metal uptake, but it can also inhibit metal uptake by forming a metal-organic acid complex outside the root preventing its uptake (Sheoran *et al.*, 2011).

Plants can also solubilize metals by exuding H^+ ions through their roots to acidify the rhizosphere. Protons can displace metal cations adsorbed to soil particles and increase metals in solution (Alford *et al.*, 2010). Roots exudates can lower the rhizosphere soil pH about one or two units over that in the bulk soil (Ali *et al.*, 2013). Another strategy is secretion of enzyme root reductases. In fact, some plants can reduce Fe^{3+} or Cu^{2+} under low Fe and Cu supply to increase plant uptake of these metals, for instance, ferric reductase oxidase 2 (FRO2) is capable of reducing Cu^{2+} during Cu deficiency (Sheoran *et al.*, 2011; Ryan *et al.*, 2013).

Mechanisms of metals and metalloids uptake, translocation and tolerance

Uptake of metals and metalloids into root cells involves transportation of metals and metalloids from the soil solution (metal bioavailable) into the roots (Tak *et al.*, 2013). This transport is mediated by transmembranes proteins specialized, or H^+ coupled carrier proteins present in plasma membrane of the root (Ali *et al.*, 2013; Tak *et al.*, 2013). These transmembranes proteins are required because metal ions are charged and cannot move freely across the cellular membrane (Tak *et al.*, 2013). Non-essential metals may effectively compete for these transmembranes transporters because they have analogous oxidation states and ionic radii (Ali *et al.*, 2013).

After entering roots, metals ions can either be stored in this plant tissue or translocated to the shoots primarily through xylem vessels where they are mostly compartmentalized in vacuoles (Ali, *et al.*, 2013). This compartmentalization in vacuoles is part of the tolerance mechanism that metal and metalloids hyperaccumulation plants have (Ali, *et al.*, 2013). This mechanism operates along with reduction of uptake into the cytosol by entrapment in the apoplasmic space,

chelation of metals in the cytosol by a range of ligands or efflux from the cytosol into the apoplastic space (Hall, 2002).

Metallophytes plants

Some plants are more tolerant than other to metals presence, for instance the metallophyte plants.

Metallophytes plants are ecophysiological adapted to thrive in metalliferous soil (Ali *et al.*, 2013), and have the unique natural capacity to concentrate essential and non-essential elements from the metalliferous soil, through an extensive root system that spreads and cover a large area in the soil and functions as a solar-driven pump (Sheoran *et al.*, 2011). Metallophytes plants are concentrated in the Brassicaceae family, which is seen as botanical curiosities (Ali *et al.*, 2013). Their use is a captivating idea for phytoremediation of metals and metalloids, alone or combined with microorganisms (Ali *et al.*, 2013).

Metallophytes plants can be divided into three categories: *metal excluders*, *metal indicators*, and *metal hyperaccumulators* (Ali *et al.*, 2013).

Metal excluders include plants that accumulate metals in their roots, restricting their transport into aboveground structures. These plants have low capacity for phytoextraction but have potential for phytostabilization (Sheoran *et al.*, 2011).

Metal indicators do not restrict metal transport to aboveground structures of the plant and accumulating metals in these structures (Sheoran *et al.*, 2011).

Metal hyperaccumulators are species of plants that can accumulate a very high concentration of metals in their aboveground tissues, including some toxic metals, to levels that far exceed the soil levels or those found in the nearby growing non-accumulating plants (Sheoran *et al.*, 2011). At the end of the last century about 24 copper hyperaccumulating species (e.g. *Ipomoea alpine*), belonging to several families were known (Kabata-Pendias, 2011). Three basic characteristics distinguishable hyperaccumulators from related non-hyperaccumulating plants: a strongly enhanced rate of metal uptake, a faster root-to-shoot translocation, sequester metals in leaves (Rascio and Navari-Izzo, 2011), and a bigger ability to detoxify, probably by adapting the mechanisms involved in the general homeostasis of constitutive tolerance to essential metal ions that exists in all plant species (Hall, 2002). Hyperaccumulation has been

taken as an extreme physiological response in metal tolerance (Baker, 1981). This phenomenon is interesting not only from the perspective of botanical but also as a potential application in the remediation of metal contaminated areas (White, 2012). In the case of the copper, some plant species have shown potential for its phytoextraction (natural and induced both) such as *Commelia communis* of the Commelinaceae family and, *Brassica Juncea* or *Ipomea alpina* of the Brassicaceae family (Sheoran *et al.*, 2011).

Plant mechanisms for metal and metalloids detoxification

The primary response of plants when exposed to high levels of metals/metalloids is the generation of ROS. The toxicity from this exposure can lead to generate ROS directly through Haber-Weiss reactions or overproduction of ROS, or by indirect mechanisms causing oxidative stress, include their interaction with the antioxidant system, disrupting the electron transport chain (Yadav, 2010), disturbing the metabolism of essential elements (e.g. the replacement of an essential metal in metalloproteins for the metal toxic) or enzyme inhibition (Van Assche and Clijsters, 1990).

In addition, one of the most deleterious effects induced by this exposure in plants is lipid peroxidation, which can directly cause membrane deterioration (Yadav, 2010). However, plants have developed potential antioxidant defences to combat the metal toxicity, e.g., plants can produce low molecular weight biological thiols (glutathione and cysteine) that show high affinity for toxic metals and are crucial for detoxification of metals (Yadav 2010). But if the plant cannot increase the activity of antioxidative defences (detoxification and repair mechanisms) in an exposure situation to the toxicity, uncontrolled oxidation reactions will lead to oxidative stress, with serious damage to the cell (Dietz *et al.*, 1999).

Microorganisms-assisted phytoremediation of metals/metalloids

Plants growing in contaminated soils harbor a diverse group of microorganisms that are capable of tolerating the contamination and providing several benefits to the soil and to the plant (Rajkumar *et al.*, 2012). This microorganisms present in the rhizosphere can positively affect plants by

improving growth, stimulate plant acquisition and recycling of nutrients, increasing plant tolerance to environmental stresses, control pathogens (Glick 2010; Rajkumar *et al.*, 2010; Aafi *et al.*, 2012).

These microorganisms in rhizosphere deserve special attention because they can directly improve the phytoremediation process by changing the bioavailability of metals in soil by altering soil pH, catalyzing redox transformations and releasing chelators (e.g. organic acids, siderophores) or immobilize them and reduce their bioavailability (Sheoran *et al.*, 2011; Rajkumar *et al.*, 2012; Ali *et al.*, 2013) (Fig. 3).

Fig. 3 summarizes the effect of microbial metabolites/actions on metals/metalloids mobilization/immobilization and/or its uptake by plants.

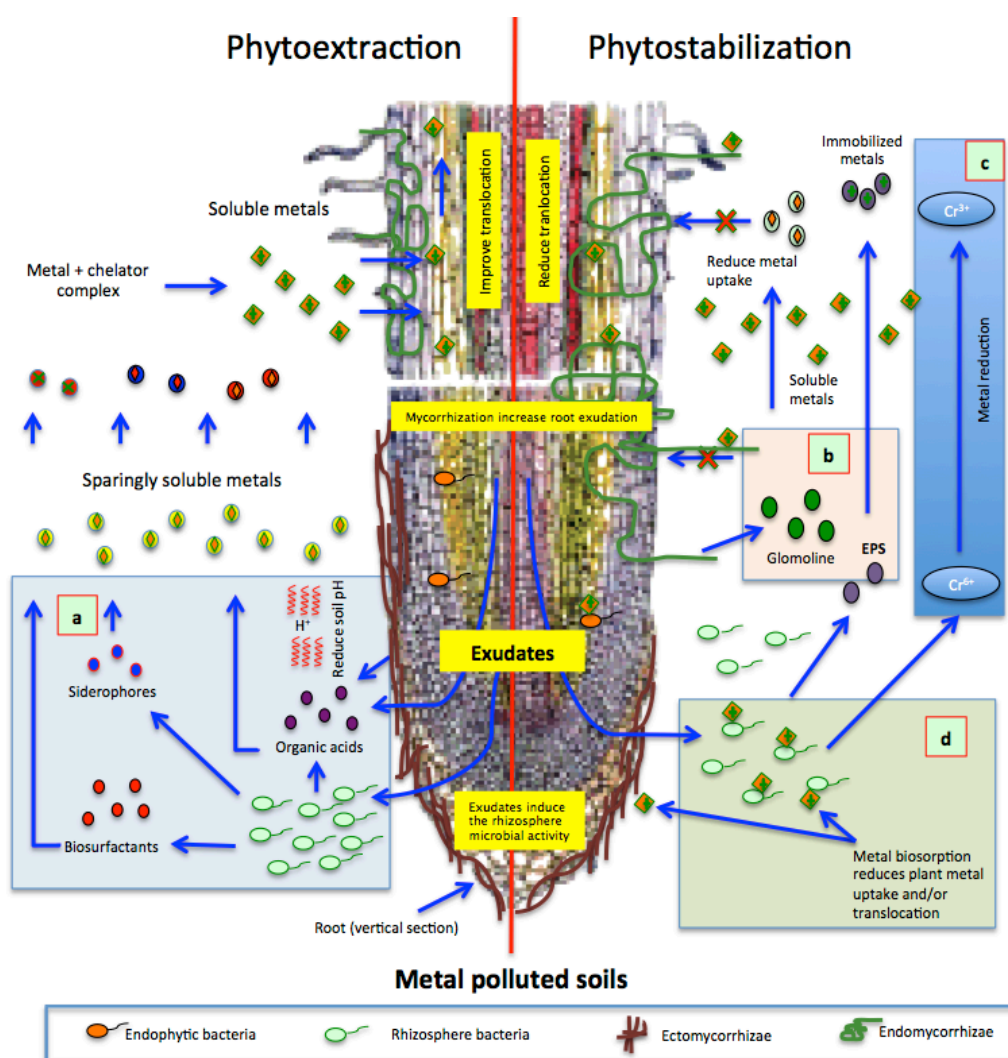


Figure 3 Plant-associated microorganisms of rhizosphere in the remediation process in metals/metalloids contaminated soils by raising metal mobilization/immobilization. “(a)

plant-associated microbes improve plant metals/metalloids uptake by producing metal mobilizing chelators. Plant associated microbes can may reduce plant metals/metalloids uptake and/or translocation through (b) producing metal immobilizing metabolites, (c) metals/metalloids reduction and/or (d) metals/metalloids biosorption. Abbreviations: extracellular polymeric substances (EPS)" (modified image by Inês PFM Montenegro from Rajkumar *et al.* (2012)).

The microorganisms in rhizosphere also have an important role in phytoextraction of metal/metalloids from soils/sediments (Fig. 3). In fact the presence of microbial symbionts such as rhizosphere bacteria can affect accumulation of metals in plants, e.g. De Souza *et al.* (1999) found that when bacteria were inhibited with antibiotics, plants (*Scripus robustus* and *Polypogon monspeliensis*) accumulated lower concentration of Se and Hg.

Biostimulation/bioaugmentation

Stimulation of naturally occurring microorganisms in symbiotic association with the plant (biostimulation) or inoculation of pollutant-resistant bacterial strains or cultures (bioaugmentation) can be a successful approach for remediation of contaminated soils, specifically with organic contamination (Gerhardt *et al.*, 2009), as mentioned in previous sections. But, metal phytoremediation can also be optimized at a certain extent when associated with biostimulation/bioaugmentation technologies (Shilev *et al.*, 2009; Wu *et al.*, 2010). In fact, recent studies have shown autochthonous bioaugmentation could increase the potential of a saltmarsh plant, *P. australis*, to phytoremediate cadmium (Oliveira *et al.*, 2014) or copper (Nunes da Silva *et al.*, 2014) from contaminated saltmarsh sediments. The relatively limited number of studies on the effects of the rhizobacteria on metal uptake in estuarine ecosystems, especially saltmarshes, suggests that it is a subject in need of greater attention.

3.3. Saltmarshes and phytoremediation of metals/metalloids

When accumulated in saltmarsh sediments, metals/metalloids can become adsorbed to the sediment constituents. The metal bioavailable fraction is the one that is uptake by plant roots and leading to metal accumulation in plant

belowground tissues or translocated to plant aboveground organs (Caçador and Duarte, 2011).

To understand the role of halophytes on the remediation of metals in the salt marshes we need to contemplate not only the amount of metal and metalloids in sediment, but also the speciation of the metal and metalloids (Caçador and Duarte, 2011), as mentioned in previous sections. The capacity of halophyte plants to influence the concentration and speciation of metals in the rhizosediment is well documented in different studies (Almeida *et al.*, 2004; Mucha *et al.*, 2005; Almeida *et al.*, 2006a). In this context, for instance, sea rush *J. maritimus* can change metal mobility and availability in rhizosphere not only by changing oxidative conditions of rhizosediments but also by the exudation of organic acids (malonate and oxalate) (Mucha *et al.*, 2005).

Halophytes plant species are ideal candidates for phytoextraction and/or phytostabilization of metal/metalloids polluted soils/sediments, including those affected by salinity such as saltmarshes located in polluted estuaries (De Lange *et al.*, 2013; Curado *et al.*, 2014; Anjum *et al.*, 2014). For instance, some salt marsh plants species such as *P. australis* (Oliveira *et al.*, 2014), *S. maritimus* (Almeida *et al.*, 2006a), *H. portulacoides* (Almeida *et al.*, 2009), *S. alterniflora*, *Sarcocornia perennis*, *J. maritimus* (Nunes da Silva *et al.*, 2014), *Triglochin maritime* (Castro *et al.*, 2009), have been studied for metals / metalloids phytoremediation.

4. Organic pollutants

Are considered as organic pollutants: phthalic esters (PAEs), polybrominated diphenyl ethers (PBDEs) polycyclic aromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethylbenzene, xylene), petroleum hydrocarbons (PHC), polychlorinated biphenyls (PCBs), chlorinated solvents, explosives, dyes, pharmaceuticals and personal care products, phenolic compounds, and pesticides (Chen *et al.*, 2013; Dordio and Carvalho, 2013).

Generally, for phytoremediation of organic pollutants in soil two approaches may be applied. First, *direct phytoremediation* (Alkorta and Garbisu,

2001), which consists in the uptake directly by plants and sequestration or degradation of pollutants within the plants (Chen *et al.*, 2013). Second, *phytoremediation ex-plant* (Alkorta and Garbisu, 2001) consists in organic pollutants degradation made by enzymes secreted by the plants or by the microbial community living in plants' rhizosphere (Chen *et al.*, 2013).

So, organic contaminants can be uptake by plant (from soil) followed by metabolism (phytodegradation) or plant-assisted microbial degradation by microorganisms living in rhizosphere (rhizodegradation) can occur. However, this uptake from soil is limited by the compound availability and uptake mechanisms (Salt *et al.*, 1998). Organics pollutants can also be immobilized by plant roots through sequestration processes (phytostabilization) (Reichenauer and Germida, 2008) and can be removed when the plant biomass is removed from a site contaminated for incineration (Gerhardt *et al.*, 2009). Those compounds having low molecular weight can be removed from soil and released to the atmosphere through leave stomata, via evapotranspiration process (phytovolatilization) (Gerhardt *et al.*, 2009).

Plants metabolism of organic compounds is a vital phytoremediation process, and this metabolism appears to follow detoxification or elimination metabolic processes, which are in tandem with designated the "green-liver" model (Burken, 2003). The metabolism of xenobiotics in plant also works like drug metabolism in liver human, in three phases, however, the only discrepancy is in *Phase III*, xenobiotics are not excreted as in human, but modified xenobiotics are sequestered in the vacuole or on the cell walls (Reichenauer and Germida, 2008).

4.1. Petroleum

Petroleum is a complex mixture of hydrocarbons and other organic compounds, and this is probably the most ubiquitous organic compound found in soil (Van Hamme *et al.*, 2003; Huesemann, 2004).

During the refining process, crude oil is transformed into useful products that are characterized by different petroleum hydrocarbons and can be grouped into four broad categories: " saturates (branched, unbranched, and cyclic

alkanes), aromatics (ringed hydrocarbon molecules such as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs), resins (polar oil- surface structure dissolved in hydrocarbons) and asphaltenes (dark-brown amorphous solids colloiddally dispersed in saturates and aromatics) ” (Macaulay and Rees, 2014).

Within the same hydrocarbon group generally the compounds exhibit analogous susceptibilities to biodegradation (Huesemann, 2004). The susceptibility of crude oil components to biodegradation may be placed in the following order: alkanes > light aromatic (MAHs) > cycloalkanes > heavy aromatic (PAHs) > asphaltenes (Van Hamme *et al.*, 2003).

The conventional techniques: thermal desorption, soil washing and solvent extraction are successful in removing petroleum hydrocarbons from soils, however, these techniques not only destroy the soil structure but also render the soil biologically impoverished, sometimes even completely sterile. In addition, its application is expensive (Huesemann, 2004).

By contrast, one of the biological methods to clean up the soil that has been explored is the use of “oil-eating” or “oil-loving” (oleophilic) microorganisms, the designated *bioremediation* (Macaulay and Rees, 2014). This method can be a significant improvement for soil quality, because it reduces hydrocarbon pollutant levels sufficiently, with reduced costs (Huesemann, 2004). Naturally occurring bioremediation is also known as *bioattenuation*, in which the petroleum hydrocarbons are converted by naturally occurring or indigenous soil microorganisms into carbon dioxide, water, bacterial cells (biomass), and humus materials (Huesemann, 2004).

But bioattenuation is usually too slow to meet the immediate need of the environment after an oil spill. However, biostimulation and bioaugmentation can be a strategy to avoid this (Macaulay and Rees, 2014).

The rate, extent, and speed of hydrocarbons degradation by microorganisms are influenced by numerous factors, including soil properties, such as moisture content, age, aeration, nutrient status, pH, humidity,

temperature, wastes characteristics (concentration, and molecular structure of hydrocarbon compounds or classes), type and the metabolism of the microorganisms (Van Hamme *et al.*, 2003; Huesemann, 2004). However, petroleum biodegradation can also potentially be inhibited by high hydrocarbon concentrations in soil, excessive nitrogen fertilization or presence of high salts and metals concentrations (Huesemann, 2004).

A considerable number of works has demonstrated the contribution of plants to remediation of soils contaminated with organic chemicals through the “rhizosphere effect” – the area around the root where there is an increase in microbial biomass and activity (Arthur *et al.*, 2005).

4.2. Phytoremediation of organic pollutants

Organic contaminants can be extracted, degraded, volatilized or stabilized, however, this depends on the compounds, its chemical nature, external temperature, type of plant and stage of growth of the plant (Nnadi and Chigbo, 2014).

As opposition to inorganic, organic contaminants can be degraded via enzymatic, modification into relatively non-toxic compounds such as carbon dioxide (CO_2), nitrate (NO_3^-), chlorine (Cl^-) and ammonia (NH_4^+) (Meagher, 2000). In the degradation process operate contaminant – degrading enzymes (e.g. peroxidases, dioxygenases, P450 monooxygenases, laccases, phosphatases, dehalogenases, nitrilases and nitroreductases) (Fig. 4). These enzymes can be found in plant, fungi, endophytic bacteria, and root-colonizing bacteria (Reichenauer and Germida 2008; Gerhardt *et al.*, 2009).

Using naturally occurring microorganisms in symbiotic association with the plant and purposeful stimulation via inoculation of the bacterial culture (bioaugmentation) more successful approach into remediation of contamination can be obtained, specifically for organic contamination (Gerhardt *et al.*, 2009).

The process of bioremediation depends on the metabolic capacity of the microorganism to detoxify or transform the contaminants bioavailable (Megharaj *et al.*, 2011). It is contemplated in the literature, that biodegradable fraction of

the organic contaminants in soil is the fraction that may be easily desorbed to or is desorbed from soil and present in the soil solution, however, it is adverted in some studies also that sorption is not limiting the biodegradation of either aromatic or aliphatic hydrocarbons (Stroud *et al.*, 2007).

Fig. 4 represents the “rhizosphere effect”, enzymatic and microbial activities responsible for the enhanced remediation of organic pollutants in rhizosphere.

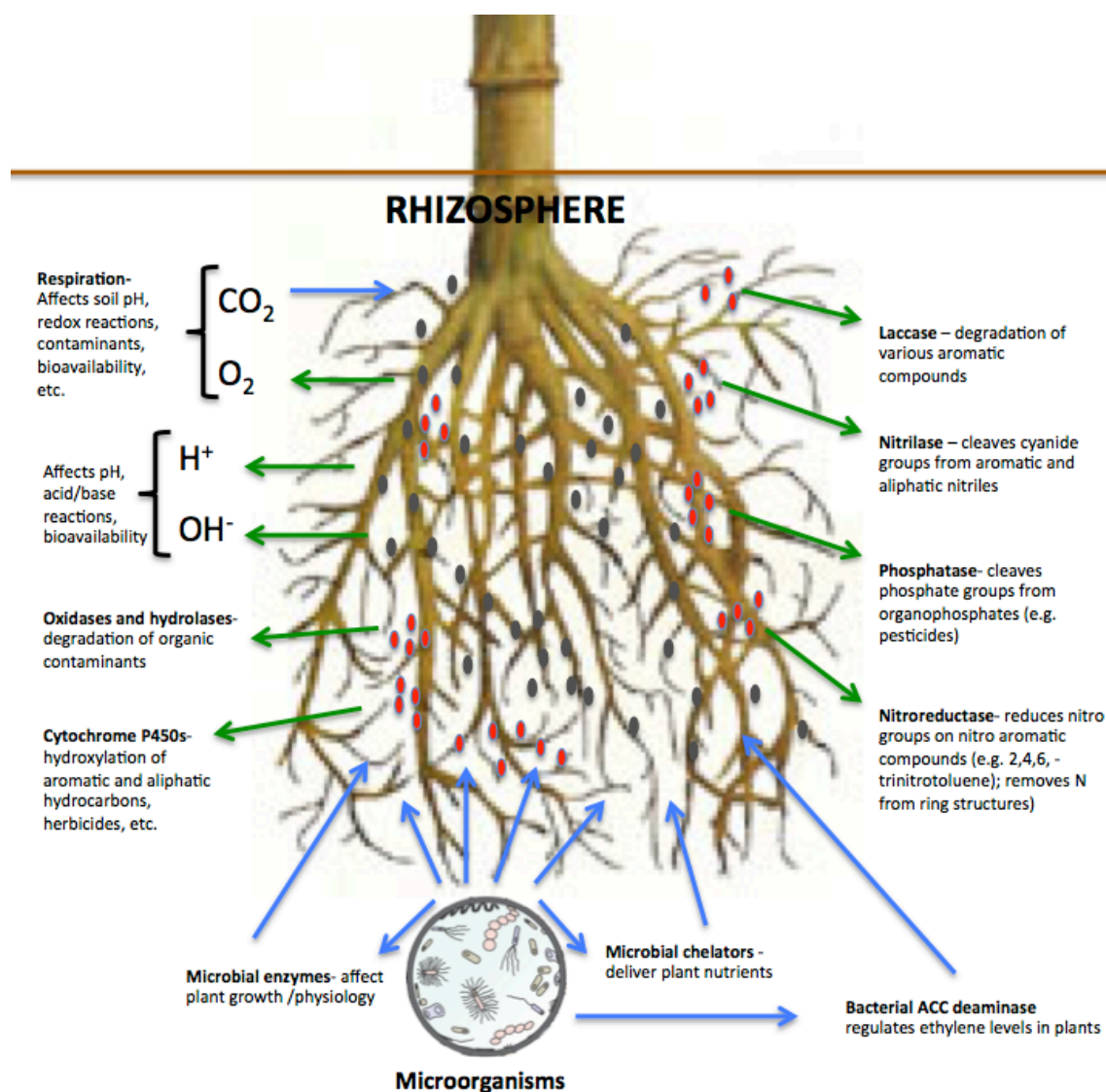


Figure 4 Schematic representation of the enzymatic and microbial activities for remediation of organic pollutants in rhizosphere (modified image by Inês PFM Montenegro from Abhilash *et al.* (2009)).

The ability of degradation by microorganisms is the key of the remediation process of hydrocarbons in soils, which is controlled by hydrocarbon physico-chemical properties, environmental conditions, soil properties, bioavailability and

the presence of degrading active microorganisms (Stround *et al.*, 2007). The microbial biodegradation of organic pollutants may occur by anaerobic or aerobic metabolism, however, anaerobic metabolism has been used as the most common method in bioremediation (Kang, 2014), however, according to others authors the most efficient in biodegradation is aerobic metabolism.

This microbial capacity to degrade may be driven by energy needs. The bacterial organisms may have two energy sources, sun energy (*phototrophs*) or chemical energy (*chemotrophs*) generated from the oxidation of chemical compounds, which can be used as substrate (*organotrophs*) and which resulted in the formation of high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond. This energy is stored in these compounds as high-energy phosphate bonds and can be used as energy sources to synthesize the new complex organic compounds needed for maintenance of basic cell physiological activities (Jurtshuk, 1996).

The soil properties affect soil-contaminant interactions and can change microbial degradation. For instance, the interactions between hydrocarbons and mineral surface (clay, silt and sand) are only significant when the organic matter content is <0.1%, and the sorption of hydrocarbons within the hard carbon phase of organic matter (glassy) is considered by irreversible sequestration (Stround *et al.*, 2007).

As already mentioned biostimulation and bioaugmentation can be used in the bioremediation of organic pollutants. The process of the bioaugmentation, as it applies to remediation of petroleum hydrocarbon contaminated soil, involves introducing into the system microorganisms selected in laboratory culture with the ability to degrade various chains of hydrocarbon into a contaminated system, and these microorganisms cultures may be derived from the contaminated soil or from a stock of microorganisms that have been earlier isolated with potential for degrade hydrocarbons (Sarkar *et al.*, 2005).

4.3. Saltmarshes and phytoremediation organic pollutants

Petroleum compounds represent one of the most common groups of pollutants in coastal environment including estuaries and most of them are known to be persistent and toxic (Mucha *et al.*, 2011). Microbial communities have important roles in saltmarsh ecosystems, such as recycling of nutrients and/or degradation of organic contaminants (Mucha *et al.*, 2013) and rhizosediments characteristic has a strong influence on hydrocarbons bioavailability (e.g. Ribeiro *et al.*, 2013b).

A study conducted by Ribeiro *et al.* (2014), revealed that salt marsh plants (*J. maritimus* and *P. australis*) and their associated microorganisms present a potential for rhizoremediation of petroleum hydrocarbons in contaminated saltmarsh sediments.

5. Co-contamination

Co-contaminated soil/sediments can be delineated as a simultaneously contaminated soil/sediments with pollutants of different nature (Almeida *et al.*, 2009).

Phytoremediation of co-contamination is still poorly understood, but it is a very relevant area of research because pollutants usually appear not singly but together in many soils/sediments and waters (Pilon-Smits, 2005; Nnadi and Chigbo, 2014; Agnello *et al.*, 2015). The most frequent combination of pollutants (co-contaminants) are metals/metalloids plus mineral oils, affecting 35% and 24% of European soils (Van Liedekerke *et al.*, 2014).

The effect of co-contamination on fauna and flora is difficult to predict (Zhang *et al.*, 2011). Though the approaches for the remediation of these sites are different (Nnadi and Chigbo, 2014) phytoremediation could be an excellent alternative to conventional methods in the remediation of inorganic (metals/metalloids) or organic contamination (Zhang *et al.*, 2011).

The co-contamination of soil might affect the remediation potential of plants (Almeida *et al.*, 2008), may interfere in growth and biomass of the plant (e.g. Zhang *et al.*, 2011), because the different compounds can interact with themselves (formation of soluble/insoluble inorganic-organic complexes) and /or with plants (modify plant growth and metabolism) and their rhizosphere (alteration in biological and physic-chemical conditions) (Guittonny-Philippe *et al.*, 2015).

5.1. Impact of organic pollutants in metals/metalloids phytoremediation

Metal uptake by plants depends not only of plant species (e.g. Liu *et al.*, 2010) and sediment characteristics (e.g. Almeida *et al.*, 2004) but also on the simultaneous presence of pollutants of different nature (e.g. Almeida *et al.*, 2008).

The interaction of metal/metalloids and organic pollutants could influence metal uptake and accumulation by plants, for instance, Almeida *et al.* (2008) reported that PAHs increased the soluble copper fraction, increasing copper uptake by the saltmarsh plant (*H. portulacoides*) in elutriate solutions. Zhang *et al.* (2011) verified when PAHs were appended to cadmium resulting in improved growth of the *Juncus subsecundus* and in increased cadmium uptake by the plants.

Another example, the presence of the high level of pyrene in soil solution inhibited the ability of copper phytoextraction by *Zea mays L.* (e.g. Lin *et al.*, 2008). Batty and Anslow (2008) verify, when comparing the behavior of two plants species (*Brassica juncea* and *Festuca arundinacea*) in a co-contaminated environment (zinc plus pyrene), that both species accumulated zinc in the presence of pyrene, but the growth of *Brassica juncea* was significantly decreased when contaminant were supplied in combination.

Simultaneous presence of metal/metalloids and organic pollutants may induce a higher toxicity (Chen *et al.*, 2004), or reduce the toxicity (e.g. Wetzal *et al.*, 1994), although it is known that the effects may not be the same of each pollutant individually (Almeida *et al.*, 2009). This simultaneous presence may

have synergistic or antagonistic toxicity effects in the physiology of plant, for instance, the synergistic effects were noted on photosynthesis and growth of the *Lemna gibba*, when oxygenated PAH (1,2-dihydroxyanthraquinone) was added together with copper (Babu *et al.*, 2001), but the opposite (antagonistic effect) was verified in the growth of *Triticum aestivum* when fluoranthene was added to the metals solution (lead and copper) (Wetzel *et al.*, 1994).

Sun *et al.* (2014) verifies in their study that endemic plant species growing at the industrial sites can colonize and survive in soils with high levels of co-contamination (metals/metalloids and PAHs), showing high accumulation potential for various metals/metalloids and PAHs, when what normally happens is, even at low concentration, the co-contamination could strongly cause oxidative stress and cellular damage, resulting in the suppression of metal/metalloids uptake.

According to Puls *et al.* (1991) and cited by Wuana *et al.* (2014) “in co-contaminated soils/sediments, the transport of metals/metalloids may be enhanced in the presence of organic pollutants due to: (i) facilitated transport caused by metal/metalloids association with mobile colloidal size particles, (ii) formation of metal organic and inorganic complexes that do not adsorb to soil solid surfaces, (iii) competition with other constituents of waste, both organic and inorganic, for sorption sites, and (iv) decreased availability of surface sites caused by the presence of a complex waste matrix”.

5.2. Impact of metals on biodegradation of organic contaminants

Metals can affect negatively or positively organic pollutants bioremediation. For instance, in a study by Moreira *et al.* (2013) it was verified that the use of phytoremediation (with *Rizophora mangle* L.) was more efficient than the use of the intrinsic bioremediation (natural attenuation) for the biodegradation of petroleum hydrocarbons in the presence of some metals (copper, zinc, chromium, nickel).

Few reports have focused on the adverse effects of metal/metalloids on the organic biodegradation. These effects include extended acclimation periods,

reduced biodegradation rates and failure of the degradation of the target compound, under both aerobic and anaerobic conditions (Olaniran *et al.*, 2013).

The presence of metals/metalloids can lead to the toxicity and inhibition of plants and microorganisms growth, and a negative interference in a wide range of microbial processes (e.g., decomposition, methanogenesis, acidogenesis, nitrogen transformation) (Sandrin and Hoffman, 2007) that can affect the degradation of organic pollutants (Gutiérrez-Ginés *et al.*, 2015).

Metals/metalloids can inhibit organic pollutant biodegradation with different patterns (Mucha *et al.*, 2011). Metals /metalloids exert their toxic effects on microorganisms through on or more mechanisms, for instance, toxic metal cations may substitute for physiological essential cations within an enzyme, rendering the enzyme nonfunctional (Sandrin and Maier, 2003). Metal/metalloids may inhibit (by the ionic form of metal/metalloids) pollutant biodegradation through interaction with the enzyme directly involved in this process (e.g. pollutant-specific oxygenase) or through interaction with the enzymes involved in metabolism in general (Sandrin and Maier, 2003). Zhang *et al.* (2011) verify that when cadmium was present a negative effect on PAHs dissipation was observed. The presence of a metal such as cadmium can inhibit a broad range of microbial processes. In opposite, Moreira *et al.* (2013) observed that some metals (copper, zinc, chromium, nickel) had a direct and positive influence on the degradation of organic compounds derived from petroleum hydrocarbons as well as the presence of the plant.

Degradation of organic pollutants during phytoremediation process depends extensively on the presence of suitable microorganisms that are active and favorable environmental conditions. However metals/metalloids availability may have the negative impact on microorganisms and limiting their activity (Nnadi and Chigbo, 2014). For instance Almeida *et al.* (2013a) verify that the presence of copper caused a measurable decrease in the abundance of the hydrocarbon degrading microorganisms and in hydrocarbon degradation rates in estuarine sediments. Dobbler *et al.* (2000) observed that the presence of metal/metalloids (copper and arsenic), had a “drastic inhibitory effect”, decreasing the number of specific population of microbes and microbial diversity.

The effect of metals/metalloids toxicity on organic pollutant biodegradation in co-contamination environments results from the fact that metals/metalloids may be present in a variety of chemical and physical forms, namely, soil adsorbed species, soluble complexed species and ionic solutes (Olaniran *et al.*, 2013).

Several studies have shown that high levels of metals/metalloids in co-contaminated with oil may interfere in the rate of oil degradation (Mattina *et al.*, 2003; Al-Saleh and Obuekwe 2005). An enormous range of concentration has been reported to inhibit organic biodegradation. Sandrin and Maier (2003) provided in review a wide range of the concentration of metals/metalloids that could cause inhibitory effects on aerobic/anaerobic biodegradation of organic contaminants. There are also plant species like *Chromolaena odorata*, with capacity to grow in soil contaminated with oil (Anoliefo *et al.*, 2003) and degrade crude oil in co-contaminated soil without being significantly affected by the concentrations of metals in the soil (e.g. Atagana, 2011).

However, salt marsh plants and bioremediation in estuarine environment is still scarcely studied regarding co-contamination effects. In fact, little information exists about organic-inorganic interaction in phytoremediation by salt marsh plants and more is required.

5.3. Bioremediation in co-contaminated environments

Recent advance in bioremediation of co-contaminated environment have focused on the use of metal-resistant bacteria (cell and gene bioaugmentation), treatment amendments, clay minerals and chelating agents to reduce bioavailable metals/metalloids concentration (Olaniran *et al.*, 2013). The co-contamination, is a double stress imposed on the soil bacterial communities, this means that for effective in situ bioremediation of the organic contaminant, there must be metal-resistant microorganisms with appropriate degradative genes, or consortia of metal-resistant microorganisms with suitable potential catabolic capabilities (Pepper *et al.*, 2002).

Bioaugmentation studies focused on the introduction of microorganisms that has both metals resistant and capability of organic degradation (dual-bioaugmentation) could be an option (Roane *et al.*, 2001, Pepper *et al.*, 2002), and this strategy has been applied in a few studies in remediation of sites co-contaminated (e.g. Alisi *et al.*, 2009; Agnello *et al.*, 2015 and references therein).

But little information exists in this area of research and more investigation is required.

6. Aim of the work

Present work aimed to investigate, under controlled but environmentally relevant conditions:

- (i) the effects of the presence of an organic contaminant (petroleum hydrocarbons) on copper remediation potential of the saltmarsh plant (*Juncus maritimus*). As bioaugmentation can have a positive effect, in this work, an autochthonous microbial consortium resistant to Cu was prepared and inoculated in the rhizosphere of the plant to increase the number of microorganisms resistant to Cu in the area surrounding plant's roots, aiming that this could increase the remediation potential of *J. maritimus*.
- (ii) the effects of the presence of a metal (copper) on petroleum hydrocarbons remediation potential of the saltmarsh plant (*Juncus maritimus*) and their rhizospheric associated microorganisms, in a co-contaminated sediment. As bioaugmentation can have a positive effect, in this work, three autochthonous microbial consortia (resistant to petroleum, resistant to Cu and resistant to petroleum plus copper) were prepared and inoculated individually in the rhizosphere of the plant to increase the number of microorganisms resistant to the pollutants in the area surrounding plant's roots, aiming that this could increase the bioremediation potential of *J. maritimus*.

Experiments were carried out in microcosms with *J. maritimus* collected out from Lima river estuary (Viana do Castelo; 41° 41' N, 08° 51' W) together with the rhizosediment, and in a system that simulated the tidal cycle occurring in the natural estuarine saltmarshes environment. Sediment was contaminated with copper or petroleum (individually or together).

CHAPTER II

Materials and methods

1. Sampling

2. Sediment spiking

3. Microbial consortia preparation and inoculation

4. Microcosms ' experiments

5. Analytical determinations

5.1. Material preparation

5.2. Sample processing

5.3. Metal determinations

5.4. Metal availability in sediments

5.5. Total petroleum hydrocarbons (TPH) determinations

5.6. Hydrocarbon degrading microorganism abundance

6. Data analysis

Materials and methods

1. Sampling

J. maritimus with sediment attached to its roots (cubes with 20 cm of edge) was collected into plastic bag at low tide from a site located in the Lima River estuary (Viana do Castelo; 41° 41' N, 08° 51' W) in May 2014 (Fig. 5A;5B). Sediment had a low organic matter percentage (ca. 3%, Ribeiro *et al.*, 2013a).

Plants with similar vitality and growth were selected and collected. At the laboratory, sediment in contact with plant belowground structures (rhizosediment) was removed. Plant roots were washed with dechlorinated (through charcoal filter) tap water and put into a one quarter-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950) until assembling of the experiment.



Figure 5 (A) Picture of the sampling of *J. maritimus* in Lima River estuary (Picture retrieved from: Msc Thesis of Nunes da Silva, 2012). **(B)** Schematic representation of the sampling, plant with sediment attached to its roots (cubes with 20 cm of edge). Assembly made by Inês PFM Montenegro.

2. Sediment spiking

After separation from plant belowground structures, rhizosediment was homogenized (plant structures removed) and divided into 2.5 L portions of wet sediment and reserved in individual aluminum foil containers (three 2.5 L

portions of sediment per each tested treatment). Then, each sediment portion was mixed manually with Arabian Light crude oil (supplied by an oil refinery) in a concentrated of 5 mL/L_{wet sediment}. To facilitate sediment homogenization dechlorinated tap water (0.5 L) was added. To allow partial aging of the contamination and adsorption of crude oil to sediment particles doped sediments were left for 48 h at room temperature in a hood. Two sets of three 2.5 L portions of sediment each were left without petroleum contamination for control purposes.

Then, a copper saline solution was added to petroleum contaminated sediments (except to a set of three 2.5 L portions of petroleum contaminated sediment) to obtain a Cu concentration of 270 µg/g. This concentration, which corresponds to the effect range–median value (ERM) concentration, is indicated in sediment quality guidelines as the values above which adverse biological effects can be observed, in marine and estuarine sediments (Long *et al.*, 1995). Although this concentration of copper is unusual in estuaries, taking into account plant's capacity to uptake metals, namely copper, this concentration was chosen to see a clear metal uptake by *J. maritimus*. Copper was also added to one set of three 2.5 L portions of sediment that were left without petroleum contamination (a scheme can be seen in Fig. 6).

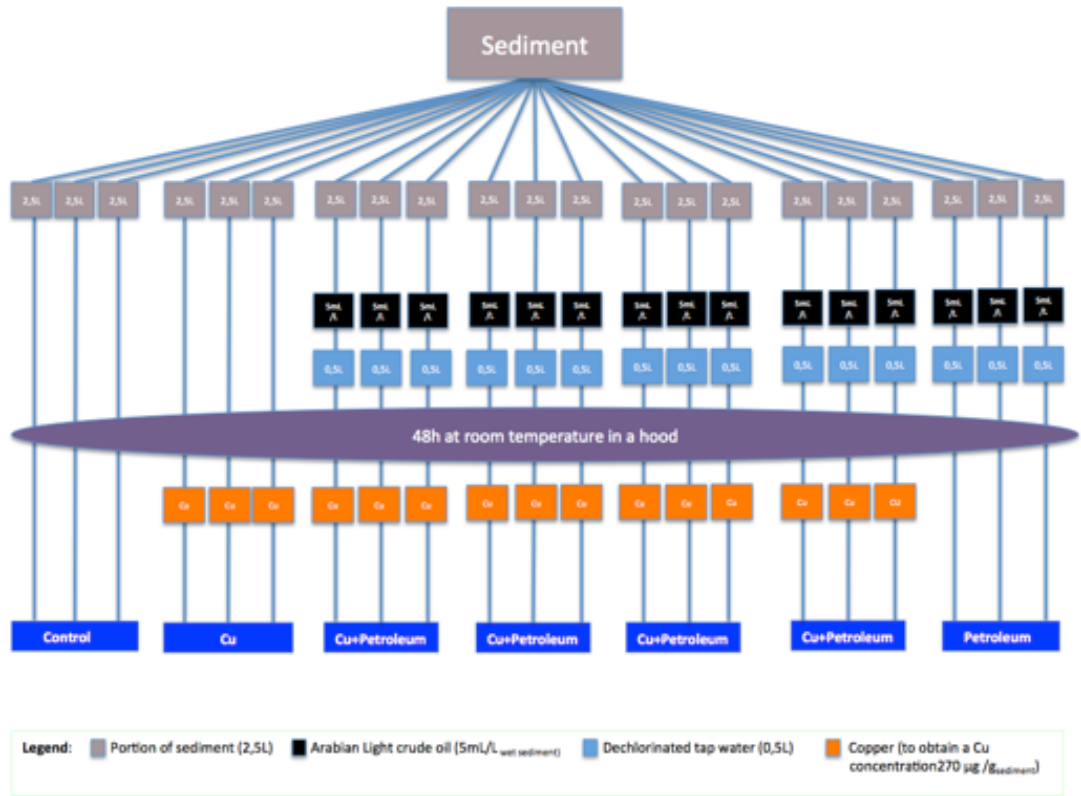


Figure 6 Flow diagram of the procedure used for sediment spiking (assembly made by Inês PFM Montenegro).

3. Microbial consortia preparation and inoculation

Three autochthonous microbial consortia (AMC) were prepared for bioaugmentation: a copper resistant enriched AMC (AM(Cu)), a petroleum resistant enriched AMC (AM(Pet)) and copper plus petroleum resistant enriched AMC (AM(Pet+Cu)).

AMC were prepared through freely adapted published procedures (Lorah *et al.*, 2008). The sediment solution (slurry) for bioaugmentation was prepared, in triplicates, with 60 mL of fresh sediments and 120 mL of estuarine water, 3.5 mL of a glucose solution (4 M) and 0,43 g of contaminant (copper or petroleum or copper plus petroleum, according to the AMC to be obtained) per replicate. This mixture was incubated for 4 days at room temperature with agitation. The 3 replicates were then mixed (making an approximate volume of 360 mL), and divided into 3 culture flasks (ca. 100 mL each). To each culture flask 100 mL of saline nutrient solution and 4 mL of glucose solution (4 M) was added and flasks were again incubated for 4 days at room temperature with agitation. Then, the 3 replicates were again mixed (600 mL) and transferred into 3 news flasks (ca. 100 mL each). The dilution step was repeated three more times. At the end all replicates were mixed again (a scheme can be seen in Fig. 7).

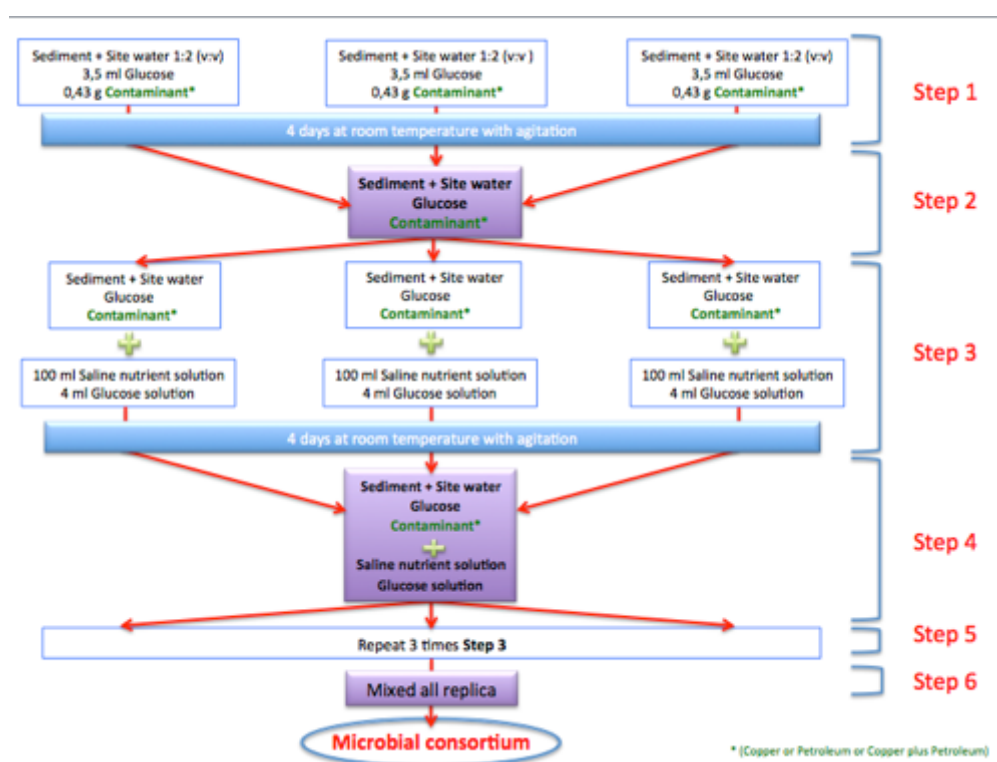


Figure 7 Flow diagram of the procedure used to produce an autochthonous microbial consortium (AMC). Assembly made by Inês PFM Montenegro.

Basically the procedure consisted in the exposure of *J. maritimus* rhizosediment to the contaminant (copper or petroleum or copper plus petroleum according to the AMC to be obtained) with sequential dilutions that allow the survival of resistant organisms.

The obtained resistant AMC (copper; petroleum; copper plus petroleum) slurry was added the vessels as described below.

4. Microcosms' experiments

Plastic vessels were assembled with a layer of pebbles with a particle size (2-4cm) at the bottom to prevent the clogging of the draining tap place at the bottom too (Fig.8A; 8B). Inside, walls vessels were covered with aluminum foil, to avoid petroleum from adhering to the plastic.

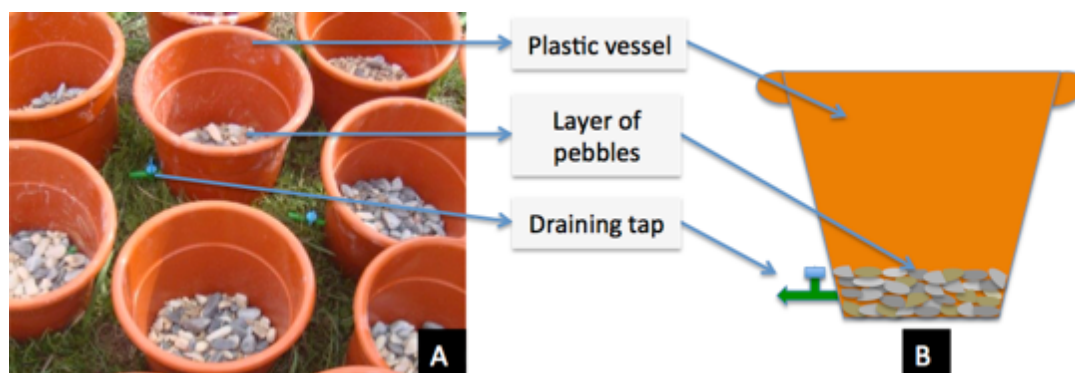


Figure 8 (A) Picture of the vessels with a layer of pebbles at the bottom; (Picture retrieved from Msc Thesis of Nunes da Silva, 2012). (B) Schematic representation of details in plastic vessels, layer of pebbles and draining tap at the bottom. Assembly made by Inês PFM Montenegro.

Rhizosediments (prepared as described above) were placed throughout 21 vessels (3 replicates of each treatment: control; doped with petroleum; doped with Cu; doped with Cu plus petroleum; doped with Cu plus petroleum and AMC resistant to Cu; doped with Cu plus petroleum and AMC resistant to petroleum; doped with Cu plus petroleum and AMC resistant to Cu plus petroleum) and plants were randomly replanted in these sediments (a scheme can be seen in Fig. 9).

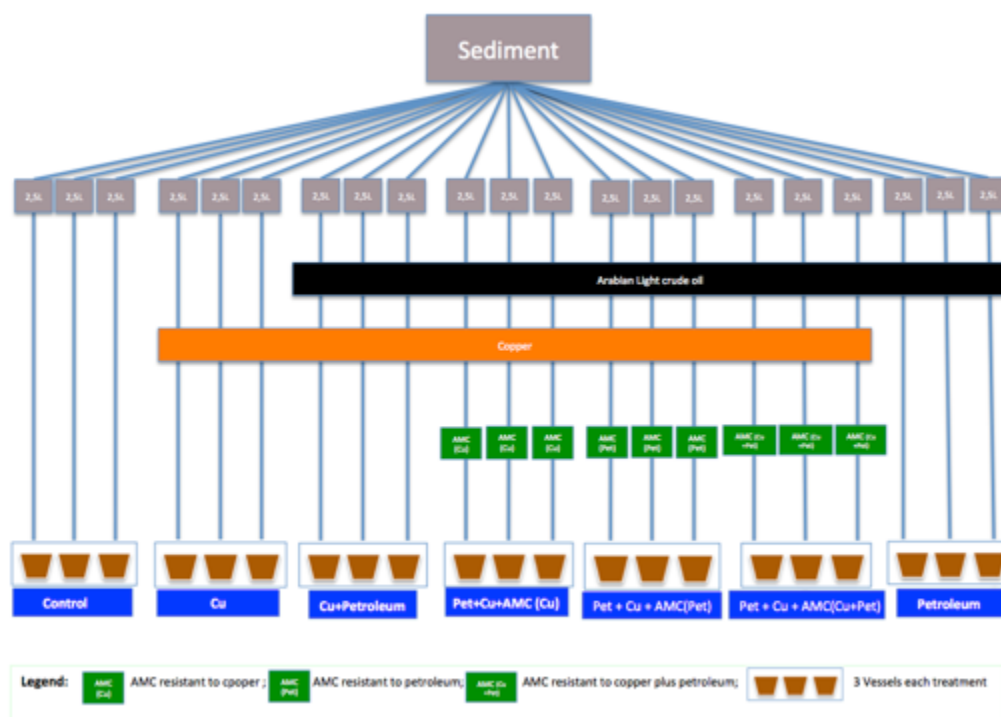


Figure 9 Flow diagram of the procedure used for the assemble experiment. Assembly made by Inês PFM Montenegro.

Vessels were randomly distributed in a greenhouse (Fig. 10). All vessels were irrigated through an automated irrigation system, adjusted to simulate tides in estuarine saltmarshes (for six hours sediments were flooded and for another six hours sediments were dry). Irrigation was carried out with a saline nutrient Hoagland solution. The irrigation solution was prepared whenever necessary by adding sodium chloride (1:100 w/v) and Hoagland solution (1:100 v/v) to 100 L of dechlorinated tap water through charcoal filter. Salinity chosen was an average value of the salinity to which the plants were exposed in their natural estuarine environment.

Vessels were kept in a greenhouse exposed to natural temperature and night/day light cycle for five months after which vessels were dismantled. The chosen experimental time (5 months) was based on previous experiments (e.g. Ph.D. Thesis, Ribeiro, 2013) in which it was observed that this was the most appropriate time to be able to observe degradation of petroleum hydrocarbons.



Figure 10 Greenhouse where plants were kept during five months with an irrigation solution through an automated irrigation system regulated to mimic the tidal cycles natural (six hours of drought and six hours of flood.) (image retrieved from Msc Thesis of Nunes da Silva, 2012).

5. Analytical determinations

5.1. Material preparation

To prevent contamination, all lab ware used for sampling and samples treatments were immersed (overnight) in a bath with 20 % (v/v) HNO_3 solution. After that lab ware was washed repeatedly with deionized water and dry in a Class 100 laminar flow hood, before use.

All reagents were pro analysis grade or equivalent.

5.2. Sample processing

After vessels dismantling sediment in contact with plant roots was separated by hand. Collected sediments from each vessel were firstly

homogenized. Then, portions of the sediments were dried at room temperature until constant weight, after which it was sieved through nylon net of 2 mm mesh to remove particles of large volume and residue of biological materials, such as roots.

Plants were washed and put to dry until constant weight, after which they were separated into roots, rhizomes, leaves and stems.

5.3. Metal determinations

Total metal (copper) was measured as before (e.g. Almeida *et al.*, 2004).

Plant tissues were firstly digested with concentrated nitric acid (HNO₃) and hydrogen peroxide (H₂O₂). In the case of sediment, digestion was carried out with concentrated HNO₃. Digestions were carried out in triplicate for each sample, in a high-pressure microwave system (MLS-1200 Mega, Milestone) in closed PTFE vessels system.

Afterwards copper was measured through atomic absorption spectrophotometry with flame atomization (AAS-F-PU 9200X, Philips), using a calibration curve obtained with aqueous standard solutions of different concentrations (0.1; 0.25; 0.5; 1; 1.5 and 3 mg/L) prepared from a copper stock standard solution of 1000 mg/L.

5.4. Metal availability in sediments

Metal availability in the rhizosediments of *J. maritimus* was estimated by sequential extraction (SE), using the method established by the Measuring and Testing Program of the European Community (Rauret *et al.*, 1999).

Aliquots of dry rhizosediment (ca. 0.50g) were treated sequentially with (a) 20.0 mL of 0.11 M CH₃COOH (Merck) solution (exchangeable fraction); (b) 20.0mL of 0.5 M NH₂OH·HCl (Ridel) solution (fraction bound to Fe and Mn oxy-

hydroxides); and (c) the metal levels in fraction bound to organic matter and sulfide plus residual fraction was calculated as the difference between the total metal levels and the sum of elemental contents extracted in the two SE fractions (a),(b). Metal was measured in each fraction by atomic absorption spectrophotometry as mentioned above. More details can be found in Almeida *et al.* (2004).

Three metals fractions were differentiated: metal exchangeable and bound to carbonates, the most bioavailable metal; metal bound to Fe and Mn (hydr) oxides; and metal bound to organic matter and sulphide and residual, the less bioavailable metal. Percentage of each fraction was calculated in relation to the total metal levels.

5.5. Total Petroleum Hydrocarbons (TPH) determinations

For TPH determination the methodology optimized by Couto *et al.* (2014) was used. The extraction step involved the addition of anhydrous sodium sulfate (Na_2SO_4) (for chemically drying the sample) and tetrachloroethylene (C_2Cl_4) (for extraction of TPH) to each sediment sample. The extraction of TPH in each sediment sample was carried through an ultrasonic bath (Elma, Transsonic 460/H model) for 30 min. After ultrasonic extraction the supernatant was centrifuged (Selecta Unitronic) at 1500 rpm for 2 min and decanted to a vial with silica gel (deactivated with 2 % of water). Non - mineral oil contaminants as animal greases and vegetable oils and other polar compounds are removed by silica gel. The vials with the mixture (extract and silica gel) were homogenized in a mechanical shaker (Unitronic) for 10 min. Before analysis, this mixture was filtered through silanized glass wool (insert into disposable pipette tips). The quantification of TPH was carried out by Fourier Transform Infrared Spectrophotometry (*Jasco FT/IR460 Plus* model) using quartz cells.

5.6. Hydrocarbon degrading microorganisms abundance

Abundance of hydrocarbons degrading (HD) microorganisms was calculated by applying a modified MPN protocol (Haines *et al.* 1996) in 96-well microtiter plates. Pre-filtered (0,2 μm) Arabian Light crude oil was used as the selective substrate for determination of total HD microorganisms. It was used medium BH supplemented with 2 % sodium chloride as a growth medium. More details can be found in Ribeiro *et al.* (2011).

6. Data analysis

For sediments copper concentrations mean values and respective errors were calculated for each treatment (i.e., for each set of three replicates, one from each vessel). For copper in plant tissues, due to the high plant natural variability, three replicates were analyzed for each vessel and then mean values and respective errors were calculated for each treatment.

Statistically significant differences between treatments were evaluated using Students'-test ($P < 0.05$).

CHAPTER III

Effect of petroleum hydrocarbons in copper phytoremediation by a saltmarsh plant (*Juncus maritimus*) and the role of autochthonous bioaugmentation

1. Results

1.1. Plant biomass

1.2. Metal levels

1.2.1. Metal levels in plant organs

1.2.2. Metal levels in sediment

1.3. Translocation Factor (TF) and Enrichments Factors (EF)

1.4. Metal fractionation

2. Discussion

2.1. Plant biomass

2.1.1. Influence of pollutants

2.1.2. Influence of autochthonous microbial consortium

2.2. Metal levels

2.2.1. Influence of pollutants

2.2.2. Influence of autochthonous microbial consortium

3 Conclusions

Effect of petroleum hydrocarbons in copper phytoremediation by a saltmarsh plant (*Juncus maritimus*) and the role of autochthonous bioaugmentation

1. Results

Results of this investigation provide information regarding the potential of saltmarsh plants such as *J. maritimus* and the microorganisms associated in phytoremediation of co-contaminated soil.

In this part of the investigation it was only used the AMC resistant to copper, being measured the metal levels in the organs of the plant, as well as metal levels in sediment and respective metal fractionation.

The experimental work for obtaining these results, featured in this chapter, is outlined in Chapter II (Materials and Methods).

1.1. Plant biomass

In the control treatment, the sediment was not doped with any of the contaminants, being exposed under the same experimental conditions as the other treatments (doped with petroleum, doped with copper, doped with petroleum plus copper). At the end of the experiment the biomass of each plant organ was obtained after drying to constant weight.

Biomass results are shown in Table 1 and were obtained by weighing, being expressed as means and standard deviation of three replicates for each treatment.

Table 1 Dry weight (g) of the plant organs (average and standard deviation, n=3) for each treatment (control; doped with petroleum (Pet); doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu))). Plant total weight per treatment is also included. Values showing

different letters indicate significant differences ($p < 0.05$, t -student) and with the same letters indicate differences not statistically significantly ($p > 0.05$, t -student).

Treatment	Biomass				
	Stems	Leaves	Rhizomes	Roots	Plant
Control	66±8 ^a	26±9 ^a	12±4 ^a	2.2±0.6 ^a	106±20 ^a
Pet	69±8 ^a	24±3 ^a	13±3 ^a	2.9±0.7 ^a	108±11 ^a
Cu	72±7 ^a	25±5 ^a	13±2 ^a	2.0±0.8 ^a	111±8 ^a
Cu + Pet	73±8 ^a	22 ±2 ^a	14±3 ^a	2.3±0.6 ^a	112±10 ^a
Cu + Pet + AMC(Cu)	83±12 ^a	29±4 ^a	14±2 ^a	2.5±0.2 ^a	128±17 ^a

Using as a reference the biomass of different organs of plants in *control* treatment (66g stems; 26g leaves; 12g rhizomes; 2.2g roots), no significant differences ($p > 0.05$) were observed when comparing these biomasses with the biomasses observed in the other treatments. However, in presence of the AMC(Cu) there was a tendency for an increase in plant biomass, being this increase reflected in the plant aboveground tissues.

Table 2 Correlation of the average biomass between several organs of the *J. maritimus*.

Treatment	Correlation of the biomass			
	Stems	Leaves	Rhizomes	Roots
Control	66	26	12	2.2
	0.39x	0.46x	0.18x	
		0.08x		
		0.03x		
Pet	69	24	13	2.9
	0.35x	0.54x	0.22x	
		0.12x		
		0.04x		
Cu	72	25	13	2.0
	0.35x	0.52x	0.15x	
		0.08x		
		0.03x		
Cu +Pet	73	22	14	2.3
	0.30x	0.64x	0.16x	
		0.10x		
		0.03x		
Cu + Pet +AMC(Cu)	83	29	14	2.5
	0.35x	0.48x	0.18x	
		0.08x		
		0.03x		

When calculating the correlation of the biomass of each organ in the same treatment (Table 2), and comparing these values with the correlation values of the other treatments, it can be seen that the relationship between the several treatments was, in general, maintained.

In Fig. 11 are displayed the percentages of the biomass of each organ relatively to the total weight of the *Juncus maritimus*. In *J. maritimus* about 85% of plant weight was localized at aboveground, wherein more than 60% were related to stems, and about 15% of plant weight was localized belowground.

Taking into account all treatments, including the control, a low range of variation in the percentages was observed for rhizomes [11-13%], leaves [20-25%] and stems [62-65%], being the roots the plant organ with less weight percentage variation [2-3%] (Fig. 11).

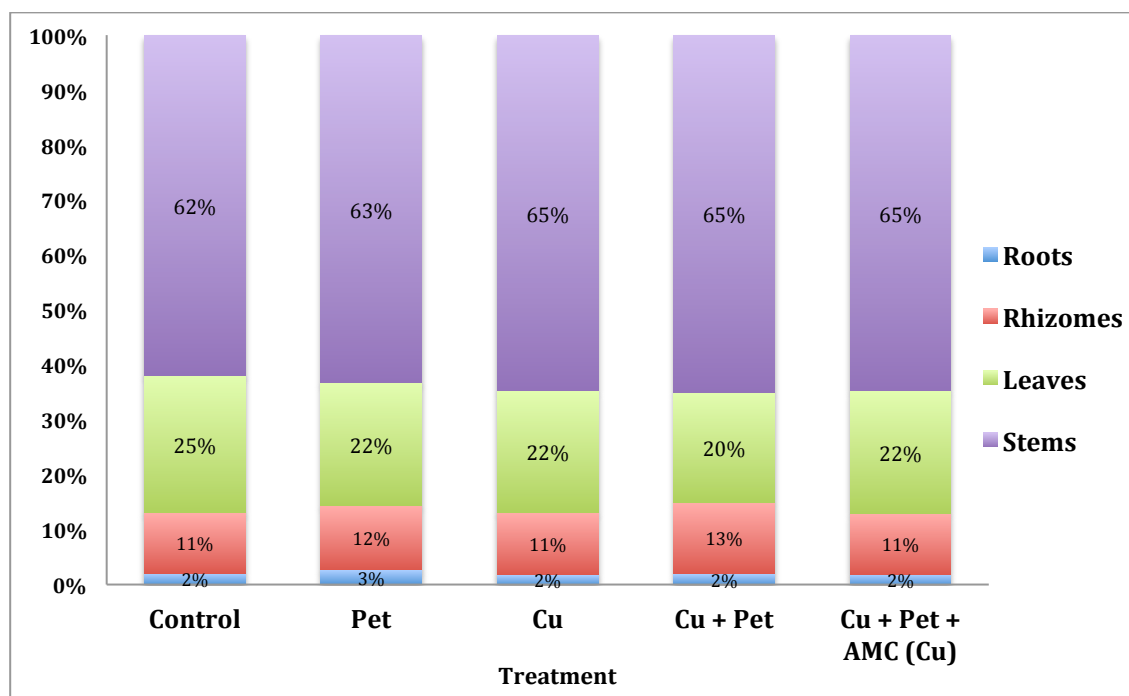


Figure 11 Percentage (%) of each plant organ weight relatively to the total biomass of *J. maritimus* in the several treatments.

1.2. Metal levels

1.2.1. Metal levels in plant organs

After five months the total copper concentrations in plant organs (roots, rhizomes, stems) were analyzed (the methodology used is described in Chapter II) and obtained values are shown in Fig. 12. The results in Fig. 12 represent the mean $\pm$ standard deviation of three replicates. Cu concentration was calculated per gram of the plant organ in the different treatments (control; doped with petroleum (Pet); doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC).

As mentioned above the *control* treatment did not have any type of doping but has been exposed to the same experimental conditions as the other treatments. No copper was measured in plant leaves because these structures are located just above the sediment surface, being very close to the sediment, and as such they may be easily contaminated during the sediment doping process. Therefore, the metal measured could be only adsorbed outside the tissue, which could interfere with the interpretation of results.

The copper levels measured in plant exposed to only petroleum were as expected identical to the ones in *control* treatment (data not show).

Each organ of *J. maritimus* showed different metal contents. Also, for the same organ, in some cases, copper concentrations varied among the different treatments.

The concentration of copper in the stems of the *J. maritimus* varied from 4.3 $\mu\text{g/g}$ to 7.0 $\mu\text{g/g}$, although differences were not statistically significant ($p>0.05$), and these copper concentrations were the lowest in the plant organs of all treatments.

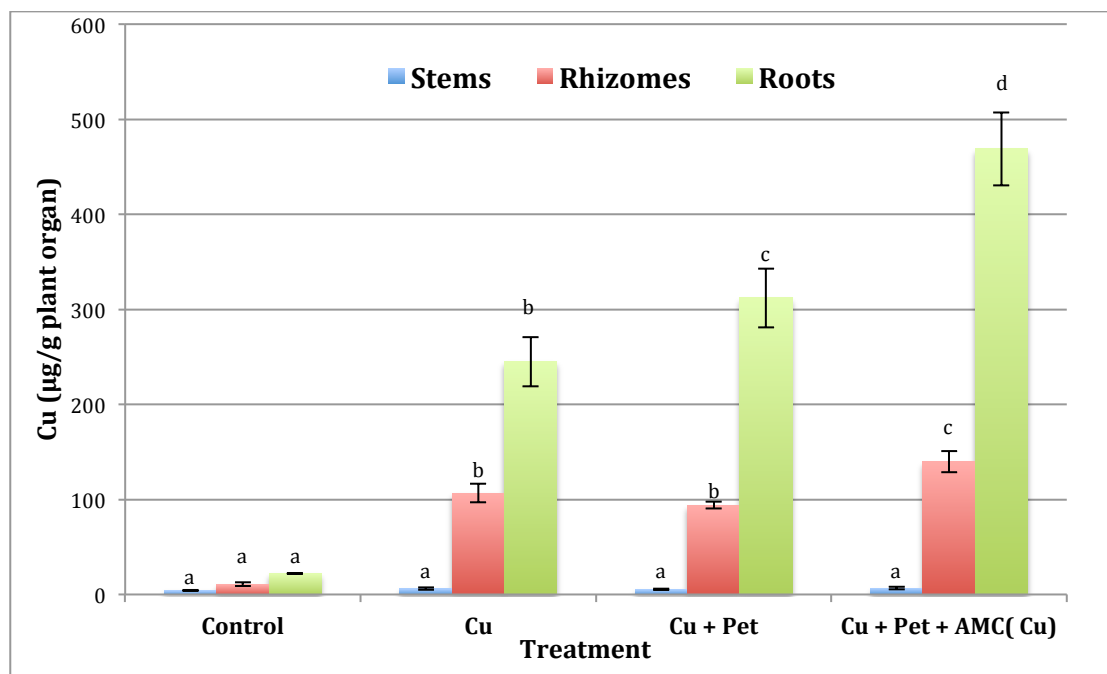


Figure 12 Total copper concentration ($\mu\text{g/g}$ _{plant organ}) in stem, rhizomes and roots of *J. maritimus*, five months after doping (mean and standard deviation, $n=3$) each treatment (doped with Cu (Cu); doped with *Cu plus petroleum* (Cu+Pet); doped with *Cu plus petroleum and AMC resistant to Cu* (Cu+Pet+AMC(Cu)). Control treatment had no doping. The values represent the means of three replicates $\pm$ standard deviation. Columns showing different letters, the differences are statistically significant ($p < 0.05$, *t*-student) and with the same letter the differences are not statistically significantly ($p > 0.05$, *t*-student).

Table 3 Correlation between treatments in copper concentration present in each organ. The (=) sign is an indication of not significantly different ($p > 0.05$) in copper concentrations between treatment (#) and (##). The (+) sign is an indication of increasing the (-) sign indicates decrease.

Treatment		Ration of copper level between treatment		
		Stems	Rhizomes	Roots
Cu [#]	Control ^{##}	=	(+) 9,7*	(+) 11,1*
Cu+Pet [#]	Control ^{##}	=	(+) 8,5*	(+) 14,1*
	Cu ^{##}	=	=	(+) 1,3*
Cu + Pet + AMC(Cu) [#]	Control ^{##}	=	(+) 12,7*	(+) 21,2*
	Cu ^{##}	=	(+) 1,3*	(+) 1,9*
	Cu+Pet ^{##}	=	(+) 1,5*	(+) 1,5*

*The value represents the ratio of metal concentration between treatment # and ##, and is calculated according to the equation (average copper level treatment # / average copper level treatment ##).

The concentrations of copper in the rhizomes of the *J. maritimus* varied from 11 µg/g to 140 µg/g and differences were statistically significant between control and the different treatments (Fig.12). In the rhizomes, the mean concentrations of copper (µg/g) were found in the order: treatment with *copper plus petroleum and AMC (Cu)* (140 µg/g) > treatment with *copper* (107 µg/g) > treatment with *copper plus petroleum* (94 µg/g) > *control* (11 µg/g). The highest Cu concentration (140 µg/g), in the treatment with *copper plus petroleum and AMC (Cu)*, was 12.7 times higher than in the *control*. In the treatment with *copper plus petroleum* copper concentration was 8.5 times higher than that observed in control treatment, but when treatment was only with *copper*, without *petroleum*, copper concentration was 9.7 times higher than in the *control* (Table 3).

The higher concentrations of copper were observed in roots, varying from 22.1 µg/g to 469 µg/g, and differences were statistically significant between control and the different treatments (Fig.12). In the roots, the mean concentrations of copper were found in the order: treatment with *copper plus petroleum and AMC (Cu)* (469µg/g) > treatment with *copper plus petroleum* (312µg/g) > treatment with *copper* (245µg/g) > *control* treatment (22.1µg/g).

The highest Cu concentration in roots (469 µg/g) was observed in the treatment with *copper plus petroleum and AMC (Cu)*. This value was 21.2 times higher than the copper concentration in plant roots of *control*. In the treatment with *copper* the value was 11.1 times the value of copper concentration in the *control*. This value becomes 14.1 times the value of copper concentration in the *control* when treatment has *copper plus petroleum* (Table 3).

When comparing the treatment with *copper* and treatment with *copper plus petroleum*, in the case of roots the value of the copper concentration in presence simultaneous of the contaminants was 1.3 times the value of copper concentration in the treatment with only *copper* (Table 3). In the case of stems and rhizomes the difference between copper concentrations observed in the different treatments were not statistically significant ($p>0.05$) (Fig. 12).

Between treatment *copper plus petroleum* and *copper plus petroleum and AMC(Cu)* the copper concentration in the rhizomes and roots were significantly different ($p < 0.05$). In *copper plus petroleum and AMC(Cu)* the copper concentration in roots and rhizomes was 1.5 times the copper concentration in treatment with *copper and petroleum* (Table 3). In stems the different between these treatments were not statistically significant ($p > 0.05$) (Fig.12).

Between treatment with *copper* and treatment with *copper plus petroleum and AMC(Cu)*, the copper concentration in the roots and rhizomes were significantly different ($p < 0.05$). In treatment with *copper plus petroleum and AMC(Cu)* in roots the value of copper concentration nearly doubles the value of copper concentration in the treatment with only *copper* and, in the rhizomes the value of copper concentration was 1.3 times the value of copper concentration in the treatment with only *copper* (Table 3).

Table 4 Correlation of the average of the copper concentration ($\mu\text{g/g}_{\text{plant organ}}$) between several organs of the *J. maritimus*.

Treatment	Correlation of the copper concentration		
	Stems	Rhizomes	Roots
Control	4.3	11	22.1
	2.5 x 5 x		
Cu	6.0	107	245
	17.8 x 40.8 x		
Cu + Pet	5.4	94	312
	17.4 x 57.8 x		
Cu + Pet + AMC(Cu)	7.0	140	469
	20 x 67 x		

After five months of exposure to different treatments, the average copper concentration varied from organ to organ of the *J. maritimus* within the same treatment (Table 4). Taking into account all treatments, the roots were the organ where the copper concentration was higher, followed by rhizomes and finally the stems, where the concentration was lower.

In the treatment *control* copper concentrations in the different organs ranged from 4.3 µg/g to 22.1 µg/g. The copper concentration had the following sequence: roots (245 µg/g) > rhizomes (107 µg/g) > stems (6.0 µg/g) (Table 4). The highest value of the copper concentration was measured in the roots (22.1 µg/g), this value was two times the copper concentration in rhizomes and five times the copper concentration in stems. In the rhizomes, the copper concentration was 2.5 times the value of copper concentration that was measured in the stems (Table 4).

The copper concentrations in the *copper* treatment ranged from 6.0 µg/g to 245 µg/g. The copper concentration had the following sequence: roots (245 µg/g) > rhizomes (107 µg/g) > stems (6.0 µg/g). The highest value of copper concentration was once again measured in the roots (245 µg/g) and was 2.3 times the concentration in rhizomes and 40.8 times the stems copper concentration. In rhizomes the copper concentration was 17.8 times the concentration of copper in stems (Table 4).

In the treatment with *copper plus petroleum*, copper concentrations ranged from 5.4 µg/g to 312 µg/g. Copper concentration follow the same sequence of the treatment mentioned above: roots (312 µg/g) > rhizomes (94 µg/g) > stems (5.4 µg/g). The highest value of copper concentration was measured in the roots (321 µg/g), being this concentration of copper 3.3 times the copper concentration in rhizomes and 57.8 times the copper concentration of stems. In rhizomes the copper concentration was 17.4 times the concentration of copper in stems (Table 4).

In the treatment with *copper plus petroleum and AMC (Cu)*, the copper concentrations ranged from 7 µg/g to 469 µg/g. The sequence of copper concentrations was the same of the treatment *control*: roots (469 µg/g) > rhizomes (140 µg/g) > stems (7 µg/g) (Table 4). The highest concentration of copper was measured in the roots (469 µg/g), which was 3.4 times the concentration of copper in the rhizomes and 67 times the concentration of copper in stems. In rhizomes the copper concentration was 20 times the concentration of copper in stems (Table 4).

1.2.2. Metal in sediment

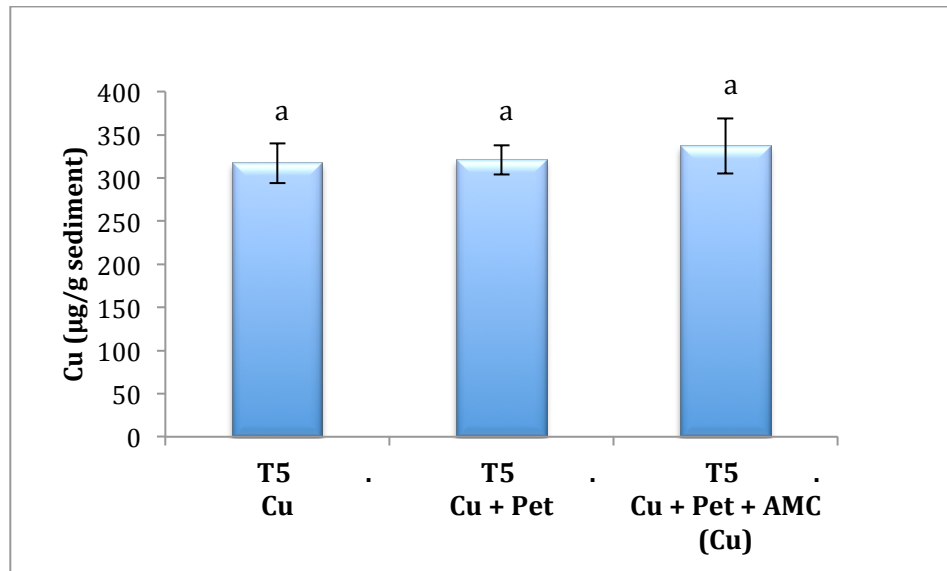


Figure 13 Total copper concentration in sediment after five months (mean and standard deviation, $n=3$), for each treatment (doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)). Columns showing different letters, the differences are statistically significant ($p<0.05$, t -student) and with the same letter the differences are not statistically significantly ($p>0.05$, t -student).

The initial concentration of total copper in the rhizosediment of *J. maritimus*, before doping with copper was 35 ± 2 µg/g sediment (result not shown). Fig. 13 shows the concentration of copper in the rhizosediments, five months after doping with copper. When observing the value of the copper in sediment (317 ± 23 µg/g in treatment with Cu; 321 ± 17 µg/g in treatment with Cu plus petroleum; 337 ± 32 µg/g in treatment with Cu plus petroleum and AMC(Cu)) and compared with initial concentration of copper in sediment, effectively doping sediment increased metal level, about 9 times. No significant differences ($p>0.05$) in the concentration of total copper in the sediment among the different treatments, after 5 months of exposure to the copper, were observed (Fig. 13).

1.3. Translocation Factor (TF) and Enrichments Factors (EF)

The translocation factor (TF), mobilization ratio of copper from belowground organs (rhizomes+roots) to aboveground organs (stems), was

evaluated (Table 5). The average translocation of copper from roots (and rhizomes) to stem, in all treatment was lower than the value observed in the control. The same value of TF is observed for the treatment with copper plus petroleum and treatment with copper plus petroleum and AMC (Cu). This value was half of the value observed for the treatment with copper only. The TF value in the control was 13 times the TF value in the treatment (copper plus petroleum) and treatment (copper plus petroleum and AMC(Cu)), and 6.5 times the TF value of the treatment with copper only.

Table 5 Translocation Factor (TF)* and Enrichments Factors (EF)**.

Treatment	Translocation Factor *	Enrichment Factor ** (aboveground)	Enrichment Factor *** (belowground)
Control	0,13	0,54	4,1
Cu	0,02	0,02	1,1
Cu + Pet	0,01	0,02	1,3
Cu + Pet + AMC(Cu)	0,01	0,02	1,8

***Translocation Factor (TF)** = concentration of metal in plant stems/concentration of metal in (rhizomes+roots);

****Enrichment Factor (EF) aboveground** = concentration of metal in plant stems/concentration of metal in sediment; *****Enrichment Factor (EF) belowground** = concentration of metal in the plant (rhizomes+roots) /

concentration of metal in the sediment.

The enrichment factor $EF_{\text{aboveground}}$ (Table 5) indicated the mobilization of copper from sediment to aboveground organ (stems). All $EF_{\text{aboveground}}$ values were below one except in the control. All treatments presented the same $EF_{\text{aboveground}}$ value. The $EF_{\text{aboveground}}$ value in control was 27 times the $EF_{\text{aboveground}}$ of the three treatments.

The enrichment factor $EF_{\text{belowground}}$ (Table 5) indicated the mobilization of copper from sediment to belowground organs (rhizomes+roots). All $EF_{\text{belowground}}$ values were higher than one, in the following sequence: *control* treatment (4.1) > *copper plus petroleum and AMC(Cu)* treatment (1.8) > *copper plus petroleum* treatment (1.3) > *copper* treatment (1.1). The value in control was 3.7 times the $EF_{\text{belowground}}$ value in *copper* treatment; 3.1 times the $EF_{\text{belowground}}$ value in treatment with *copper plus petroleum*; and 2.3 times the $EF_{\text{belowground}}$ value in treatment *copper plus petroleum and AMC(Cu)*.

1.4. Metal fractionation

Three metal fractions (*exchangeable and bound to carbonates; bound to Fe an Mn (hydr) oxides; fraction bound to organic matter and sulphite and residual*) in sediments were measured, through sequential extraction procedure, five month after contaminating the rizosediment of *J. maritimus* with copper (Table 6).

When comparing among treatments the different percentage of the metal fraction, the fraction with higher percentage was the fraction *exchangeable and bound to carbonates* [41% - 48%], followed by the fraction of metal *bound to organic matter and sulphite and residual* [26% - 33%] and fraction of metal *bound to Fe an Mn (hydr) oxides* (26%) (Fig. 14).

No statistically significant differences were observed when comparing copper concentrations in the metal fraction *exchangeable and bound to carbonates* among treatments (Table 6). In treatment with *copper* this fraction represented 48% of the total metal concentration. When comparing the percentage of this fraction in treatment with *copper* with the same fraction in the present of petroleum in *copper plus petroleum* treatment, the presence of organic pollutant (petroleum) did not affect the percentage of the copper that is *exchangeable and bound to carbonate* in sediment.

When comparing the percentage of this fraction in treatment with *copper plus petroleum* (43%) with the same fraction in the presence of AMC(Cu) in *copper plus petroleum and AMC(Cu)* treatment, the presence of microorganisms had no effect in the quantity of the copper that is *exchangeable and bound to carbonate* (Fig. 14).

Table 6 Metal fractionation in rhizosediment (average ($\mu\text{g/g}_{\text{sediment}}$) and standard deviation, n=3) for each treatment (doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu))). Cu concentrations in the different fractions are presented in $\mu\text{g/g}_{\text{sediment}}$. Values showing

different letters, indicate significant differences ($p < 0.05$, t -student) and with the same letter the differences are not statistically significantly ($p > 0.05$, t -student).

Treatment	Metal fractionation		
	Fraction exchangeable and bound to carbonates	Fraction bound to Fe and Mn (hydr) oxides	Fraction bound to organic matter and sulphite and residual
Cu	155±5 ^b	81±4,2 ^b	81±14 ^b
Cu + Pet	139±11 ^b	82±21 ^b	100±15 ^b
Cu + Pet + AMC(Cu)	145±5 ^b	94±14 ^b	119±3 ^b

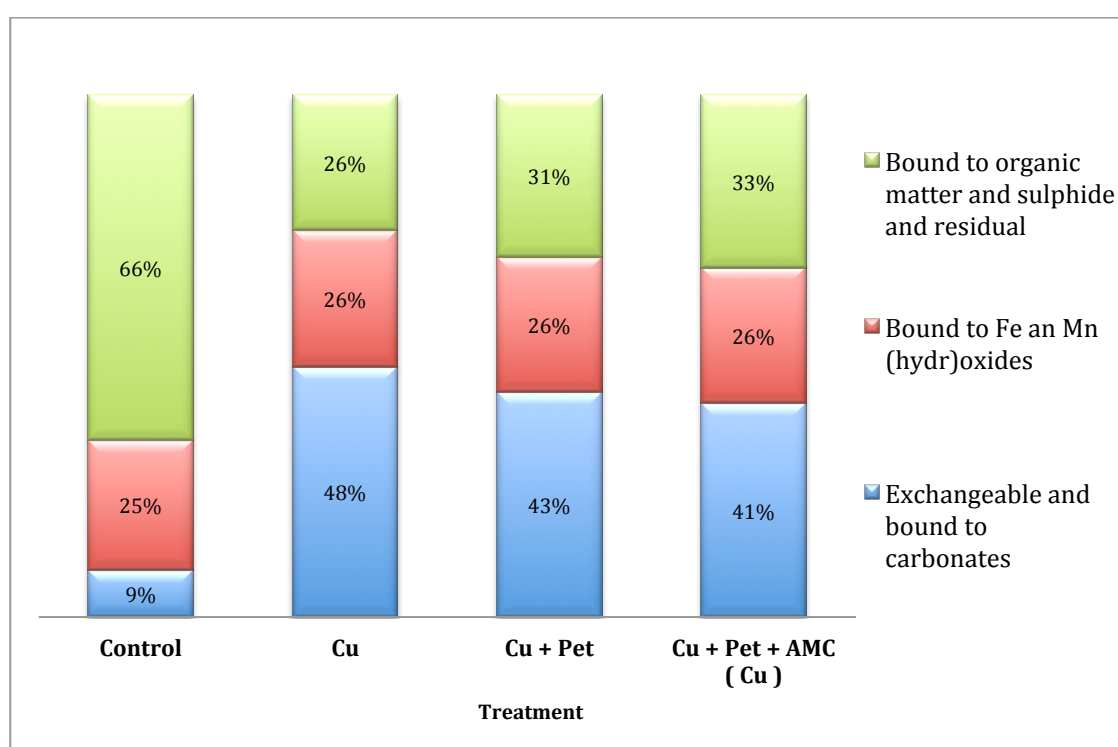


Figure 14 Percentages (%) of each metal fraction relative to the total metal concentrations present in the rhizosediment of each treatment.

Regarding the fractions of the metal that was *bound to Fe and Mn (hydr) oxides* in the rhizosediment, no statistically significant differences were observed when comparing the measured concentrations among treatments. When comparing the percentage of this fraction in treatment with *copper* (26%) with the same fraction in the presence of petroleum in *copper plus petroleum* treatment, the presence of petroleum had no effect in the percentage of the copper that was *bound to Fe and Mn (hydr) oxides*. When comparing the percentage of this metal

fraction in treatment with *copper plus petroleum* (26%) and the same metal fraction in the presence of AMC(Cu), in the *copper plus petroleum and AMC(Cu)* treatment, the presence of AMC(Cu) did not affect the percentage of the copper that is *bound to Fe and Mn (hydr) oxides* in the rhizosediment.

Regarding metal fraction that was *bound to organic matter and sulphite and residual* in the rhizosediment no statistically significant differences were observed among treatments (Table 6). When comparing the percentage of this metal fraction in treatment with *copper* (26%) with the same fraction in the presence of petroleum in *copper plus petroleum* treatment, the presence of petroleum did not affect the percentage of the copper that *bound to organic matter and sulphite and residual*. The same effect was observed in the presence of AMC(Cu) (Fig. 14).

2. Discussion

2.1 Plant biomass

2.1.1. Influence of pollutants

Plants have the capacity to degrade or sequester (e.g. vacuole) many toxic compounds when exposed to many of them (Davis *et al.*, 2002). However, the stress response of plants to the presence of pollutants can change plants capacity to control the uptake of those pollutants, increasing the uptake and sometimes causing serious problems to the viability of the plant (Almeida *et al.*, 2008).

So, plant biomass can be used as an indicator for the overall health of *J. maritimus* growing in the presence of pollutants.

Usually when it is observed an increase in the growth of plants in presence of copper, it could be related to the effects copper has on several macronutrient contents (N, P, K, Na, Ca, and Mg) (Chiago *et al.*, 2013). For example, Manivasga *et al.* (2011) suggested that lower concentration of copper (50 µg/g) increased

the nutrient (N, P, K, Na, Ca and Mg) contents of *Vigna radiate* L. resulting in an increase in leaf nitrogen, however they observed a gradual decline in plant biomass production at higher copper concentration (100-250 µg/g).

At high concentration all metals/metalloids are regarded as environmental pollutants and can have a strong toxic effect in plants, phytotoxicity resulting in chlorosis, inhibition plant growth, yield depression, which may even be accompanied by reduced nutrient uptake, disorders in plant metabolism (Guala *et al.*, 2010).

In the present work no significant differences ($p > 0.05$) in production of biomass were observed between treatments with and without copper (Table 1), indicating that, apparently, the used copper concentration (270 µg/g_{wet sediment}) did not cause inhibition or promotion of *J. maritimus* growth.

The growth of plants might be influenced not only by metals but also by toxic organic chemicals. For instance, Hechmi *et al.* (2015) verified that the biomass of *P. australis* was reduced 40% by pentachlorophenol (PCP) high level addition. However, Zhang *et al.* (2010) reported that the biomass of *Baumea articulate* (R. Br.) was significantly increased in a treatment with relatively low addition of naphthalene.

No significantly difference ($p > 0.05$) was observed in biomass of *J. maritimus* in treatment with *petroleum* only when compared with control treatment. So, the *petroleum* concentration used in this experimental condition, did not seem to be toxic for *J. maritimus* growth. However, petroleum hydrocarbons are known to be harmful to plant growth and development, this is because petroleum hydrocarbons negatively affect photosynthesis and therefore reduce nutrient assimilation and biomass accumulation (Nie *et al.*, 2011 and references therein).

No significant differences ($p > 0.05$) were either observed in *J. maritimus* growth when comparing the treatment *petroleum* plus *copper* to control. Results suggest that the biomass of *J. maritimus* was not significantly influenced by the simultaneous presence of the two pollutants and their interactions. Interactive effects (synergistic or antagonistic effects) have been reported in other works. For instance, Batty and Anslow (2008) reported that in presence of pyrene and

zinc the growth of *B. juncea* was significantly reduced. Chigbo *et al.* (2013) observed that the simultaneous presence of copper and pyrene caused a reduction in the growth of the *B. juncea*, and Zhang *et al.* (2009) showed that pyrene did not alleviate the toxicity of cadmium in *Z. mays*. But, by contrast, Lin *et al.* (2008) report an increase in shoot yield of *Z. mays* in the presence of copper and pyrene simultaneously.

These results suggest that combinations of metals /metalloids and organic pollutants may either alleviating or exacerbating effect on plant growth (Zhang *et al.*, 2012). The growth responses of the plants to the joint toxicity of metal and organic pollutants are dependent on certain factors including plant species, plant growth stage, properties and concentration of contaminants (Zhang *et al.*, 2011; Lin *et al.*, 2008), time of exposure to the pollutant (Davis *et al.*, 2002) and soil conditions such as pH and content of organic matter (Zhang *et al.*, 2012).

2.1.2. Influence of autochthonous microbial consortium

The addition of the autochthonous copper resistant microbial consortium, (AMC(Cu)) to the sediment, which was prepared for bioaugmentation, caused a slight increase in plant stems, leaves and roots biomasses and consequently in the total biomass of *J. maritimus*.

Bacteria can promote the growth of the plant by synthesizing beneficial compounds or/and facilitating the uptake of certain nutrients from soil/sediment (Burd *et al.* 2000; Çakmakçı *et al.*, 2006). Bacteria can also prevent or attenuate plant diseases (Jetiyanon and Kloepper, 2002; Guo *et al.*, 2004). For example, Lampis *et al.* (2015) observed a significant increase (35%) in the frond biomass of *Pteris vittata* grown in arsenic-contaminated soil and inoculated with arsenic resistance bacteria.

Usually microbial consortia have higher microbial variability, being more efficient than pure cultures (Gentry *et al.*, 2004) in remediation process and also in the increase of biomass. For instance, Azcón *et al.* (2010) observed increased plant biomass of *Trifolium repens* when comparing the effect of the inoculation of dual association of metal tolerant microorganisms (arbuscular mycorrhizal fungi and *Bacillus cereus*; arbuscular mycorrhizal fungi and *Candida parapsilosis*) with single inoculation of the *Bacillus cereus* or *Candida parapsilosis*.

Some experiments have, however, shown an opposite behavior after inoculation of microbial consortia in sediments. Some studies have already applied microbial consortia to enhance metal phytoextraction rate, nonetheless, it may occur a reduction in production of biomass. For example a study by Lampis et al. (2009) found that a bacterial consortium adapted to selenium increased three times the bioconcentration factor in *B. juncea* when comparing with control, but decrease phytoextraction efficiency due to reduction of biomass production.

On the other hand the use of autochthonous microorganisms can enhance the success of remediation. In fact, limiting factor for this process include the decrease of bacterial survival after inoculation due to abiotic and biotic stress factors (Gentry *et al.*, 2004).

In summary, in the present study, there were no significant changes ($P > 0.05$) in plants biomass among treatments (Table 1) indicating that none of the contaminants (alone or combined) significantly affected this parameter. However, a slight increase in plant biomass was observed when AMC(Cu) was applied to the sediment co-contaminated.

2.2. Metal levels

J. maritimus clearly uptake copper from sediment and accumulated the metal in its roots. Copper concentration in plant roots in *copper* treatment was 11 times higher than the in control (Table 3). In copper treatment, in rhizomes copper concentration was about half the copper concentration in roots (Table 3), but it was still higher than copper concentration in control. It is consensual that saltmarsh plants accumulate large amounts of metals in their belowground organs particularly in their roots, preventing metal translocation (Almeida *et al.*, 2011). Nonetheless, in rhizomes (another belowground organ of the plant) metal levels are usually considerably lower than in roots (e.g. Almeida *et al.*, 2006a).

In the present work, in treatment with *copper* only, copper translocation also occurred into the aboveground organ (stems). However, as expected, the amount of copper translocate to stem was low (Fig.12). In fact, copper concentration in stems was ca. 40 times lower than in roots and ca. 18 times lower than in rhizomes (Table 4). These results suggested that *J. maritimus* has a low translocation metal potential. Other studies have also shown this low translocation metal potential, with most of the metal burden remaining in its roots (e.g. Almeida *et al.*, 2004; 2006b). Baker (1981) reported that the restriction of copper transport to the shoot seemed to be a common feature in most plants with copper resistance. These results corroborate that metals in halophyte plants are mainly accumulated in the roots with only residual amounts translocated to aboveground organ (Weis *et al.*, 2002; Windham *et al.*, 2003; Almeida *et al.*, 2006a; Reboreda and Caçador, 2007) except in the case of elements with more mobility like Cd, Mn or Zn (Caçador *et al.*, 2000; Almeida *et al.*, 2006a).

In *J. maritimus* the aboveground biomass contributed about 85% to the total biomass for this species and belowground biomass contributed only about 15%. In fact the structure where uptake occurs and where the greatest accumulation of copper occurs (roots) is a tiny part of the whole plant (about 3% of the total plants biomass) and this obviously, has an effect in the capacity of the plants for phytoremediation/phytoextraction of metals/metalloids. For example, in a comparative study between *J. maritimus* and *P. australis* made by Almeida *et al.* (2013b), the size of the roots was one of the factors (beyond the morphology) that can determine the levels of metal uptake. This anatomical consideration is important when choosing a plant for soil/sediment remediation.

The potential of the metal/metalloids remediation by plants species can be divided into three groups: “(i) phytoextraction, is a removal process taking advantage of the unusual capacity of some plants to (hyper-) accumulate metals/metalloids; (ii) rhizofiltration, roots of metal/metalloids accumulating plants that absorb metals from polluted site and are later harvested to diminish metals in effluents; and (iii) phytostabilisation (and immobilization), metal-tolerant plants that are used to reduce metals mobility and prevents transfer to other ecosystem compartments, the metals/metalloids are stabilized in the substrate or in plants belowground structures (Abdel-Ghami *et al.*, 2007; Wenzel,

2009). Plants with both enrichment factor ($EF_{\text{belowground}}$) and translocation factor (TF) greater than one (TF and $EF_{\text{belowground}} > 1$) have the potential to be used in phytoextraction and plants with enrichment factor ($EF_{\text{belowground}}$) greater than one and translocation factor less than one ($EF_{\text{belowground}} > 1$ and $TF < 1$) have potential for phytostabilization.

As previously reported by several authors (Cheng *et al.*, 2002; Lui *et al.*, 2010; Yadav *et al.*, 2012), metal/metalloids are generally retained in greater quantities in belowground organs than in aboveground organs of plants, and *J. maritimus* is no exception to that rule. According to experimental data provided by this experiment, *J. maritimus* showed in all treatments to have a higher metal retention potential in belowground organs (rhizomes and roots). Moreover, *J. maritimus* has demonstrated a phytostabilization potential, because in all treatments the $EF_{\text{belowground}}$ was greater than one and the value of the TF was lower than one. This potential capacity of *J. maritimus* for phytostabilization, had already been observed by Almeida *et al.* (2004; 2011).

2.2.1. Influence of pollutants

As already mentioned organic pollutants can influence plant capacity to accumulate metals/metalloids in cases of co-contamination. For instance, Lin *et al.* (2008) observed a reduction in the concentration of copper in *Z. mays* when copper and pyrene were supplied in combination. In contrast, Batty and Anslow (2008) reported similar concentration of zinc in the *Festuca arundinacea* organs when zinc and pyrene were supplied in combination compared with zinc alone.

Present results showed that in the presence of petroleum, copper concentration in stems of plants from the treatment *copper plus petroleum* was statistically identical ($P > 0.05$) to that observed in plants from *copper* treatment. This result suggests that the presence of petroleum did not interfere in the translocation of copper in *J. maritimus* (Fig. 12; Table 3). For instance, the same result in the copper translocation was observed by Almeida *et al.* (2009) in *H. portulacoides* when PAHs was added to the medium.

In contrast, Batty and Anslow (2008) observed a significant increase in the concentration of zinc in shoots (stems including their appendages) of *Brassica juncea* in co-contaminated soil (zinc and pyrene) relative to soil contaminated with zinc only. Zhang *et al.* (2012) report that phytoextraction potential of cadmium by wetland plant (*Juncus subsecundus*) was enhanced by the addition of PAHs. Wang *et al.* (2014), provides an explanation for the increased in phytoextraction of cadmium when PAHs is present: the development of apical apoplastic barriers may be inhibited by PAHs, thus promoting the absorption into the body of cadmium.

The same result as for stems was observed regarding plant rhizomes. The concentration of copper was statistically identically ($P>0.05$) when comparing treatment *copper plus petroleum* with treatment *copper* (Fig. 12).

However, when petroleum was added to sediment together with copper, the copper level significantly increased ($P<0.05$) in *J. maritimus* roots compared with *copper* treatment (Fig. 12). The same result is reported by Wang *et al.* (2014), with a significant increase in cadmium concentration in *Kandelia obovata* roots in treatment with mixture of high cadmium and pyrene treatment compared to treatment with cadmium alone. Almeida *et al.* (2008) also reported a significant increase in copper concentration of *H. portulacoides* roots when copper enriched elutriate was amended with PAHs.

But Lin *et al.* (2008) observed inhibition in copper uptake by *Zea mays* L. under co-contamination of a high level of pyrene in highly copper polluted soil. These results suggest that combination of metal/metalloids and organic pollutant may have either antagonistic or synergistic effect on metal uptake by plants, depending on plant species, their growth stages, concentrations and characteristics of pollutants, and soil/sediment conditions (pH; redox potential; content of organic matter) (Wang *et al.*, 2014).

This increase of copper concentration in roots in the presence of petroleum may have an alternative explanation. Alkio *et al.* (2005) suggested that in a situation of co-contamination, the PAHs may passively penetrate the root cell membranes without any type of carrier which can therefore facilitate the penetration of metal (or metal complex) into the cell.

Metal bioavailability in sediment is an important factor for metal uptake in plants and for phytoextraction efficiency (Prapagdee *et al.*, 2013).

Plant roots can exude a variety of organic chemicals, such as organic acids, with strong binding capacities for metals (van Steveninck *et al.*, 1990). In the particular case of the *J. maritimus*, this plant can release malonate and oxalate, which may complex metals and therefore change the availability of metals in soils/sediments (e.g. Mucha *et al.*, 2005) and also may release, in a short period of time, relatively high amounts of strong copper-complexing ligands (e.g. Mucha *et al.*, 2008). It can be said that plants have an important role in metal bioavailability in co-contaminated soils/sediments.

Studies have verify that estuarine sediments can act as a significant source of copper-complexing ligands to the overlying water, which may strongly influence the biogeochemistry and cycling of dissolved copper by sediment/water exchange (Skrabal *et al.*, 1997, 2000; Shank *et al.*, 2004). Among the main sources of the organic ligands found in the sediment, Skrabal *et al.* (2000) have considered several possibilities, including exudation release by bacteria, macrofauna or other organisms; microbial metabolites (sulphide, polysulphides) and diagenetic alteration of pre-existing organic substances.

In addition, organic pollutants may cause changes in metal speciation and fractionation by altering physico-chemical sediment characteristics in the rhizosphere, through plant exudation (organic compounds released by plant roots), or microbial activity induced changes (Chen *et al.*, 2004; Lin *et al.*, 2008). For instance, in the presence of organic pollutants the exudation of organic acids may be enhanced, as reported by Phillips *et al.* (2012) in the case of *Elymus angustus* exposed to a situation of co-contamination (pyrene and phenanthrene). Almeida *et al.* (2008) report that presence of PAHs can increased copper sorption by saltmarsh plants by way of increasing copper solubility; but this phenomenon only occurred when plants were present, indicating that PAHs-copper interaction may be related to plants (Wang *et al.*, 2012).

In general, when metal is more bioavailable, for instance, bound more weakly to the sediment, a higher metal uptake can be expected. However, in the

present study metal bioavailability was lower in sediment when *petroleum* was added together with *copper*, i.e., the percentage of the metal fraction *exchangeable and bound to carbonates* decreased when compared with the same fraction in *copper* treatment (Fig. 14).

On the other hand, the values calculated of the TF and $EF_{\text{belowground}}$ (Table 5) in treatment with both contaminants (*copper plus petroleum*) indicated that presence of *petroleum* affected positively the potential of phytostabilization of *J. maritimus* when compared with the value of TF and $EF_{\text{belowground}}$ in treatment with copper only.

2.2.2. Influence of autochthonous microbial consortium

As mentioned, in the case of metal/metalloids contaminated soil/sediment phytoremediation depends mostly on the plant capacity to uptake and accumulate metals/metalloids and it is important to increase this capacity.

Bioaugmentation has several advantages over other techniques because when a specific microbial population is injected the degradation process can start immediately (Alisi *et al.*, 2009), the lag phase does not occur and microbial activity starts in the exponential phase (logarithmic phase).

To date, studies devoted to microorganism-assisted plant to increase metal phytoremediation from soil/sediment are scarce, although these studies (Lodewyckx *et al.*, 2001; Braud *et al.*, 2006; Braud *et al.*, 2009; Azcon *et al.*, 2009; Azcón *et al.*, 2010; Prapagdee *et al.*, 2013; Nunes da Silva *et al.*, 2014; Oliveira *et al.*, 2014; Arunakumar *et al.*, 2015; Lampis *et al.*, 2015) showed that the metals phytoremediation can be improved through inoculation of metal resistant microorganisms in rhizosphere. However in most of these studies specific bacterial strains were used and only a few studies used microbial consortia (e.g. Nunes da Silva *et al.*, 2014; Oliveira *et al.*, 2014).

Usually microbial consortium is more efficient than pure culture, due to the microbial diversity in bacterial consortium and may be more successful because it provides higher metabolic diversity (Oliveira *et al.*, 2014). In most of

these studies pre-selected specific bacterial strains and microbial consortia are exogenous to the sites (soil/sediments and water), which brings some disadvantages, and therefore the use of autochthonous (native/indigenous) microorganism strains or consortia from their own environment in bioaugmentation can be an advantage because the microorganisms are more likely to survive and propagate when reintroduced into the site (Alisi *et al.*, 2009).

Obtained results showed that the inoculation of an autochthonous microbial consortium resistant to copper (AMC(Cu)) in the rhizosediment of *J. maritimus*, significantly increased ($p < 0.05$) copper uptake (adsorption / absorption), increasing (about 1.5 times) copper concentration in plant roots relatively to the treatment without AMC(Cu) addition (Table 3).

Copper concentration in rhizomes also increased 1.5 times with inoculation of the AMC(Cu) (Table 3). AMC(Cu) had, therefore, a positive effect on copper uptake and improved the phytostabilization potential of *J. maritimus*. However, copper translocation was not increased in the presence of AMC(Cu), as no significant differences ($p > 0.05$) between treatments with or without AMC(Cu) were observed (Table 3).

Some studies also reported a positive effect of inoculation in the case of metal contaminated soil/sediments. Lodewyckx *et al.* (2001) observed that the nickel uptake in roots increased when *Lupinus luteis* L. exposed to nickel enriched substrate was inoculated with endophytic bacteria resistant to nickel.

Nunes da Silva *et al.* (2014) also observed an increased in cadmium uptake when the rhizospheres of *J. maritimus* and of *P. australis* were inoculated with an autochthonous microbial consortium resistant to cadmium (AMC(Cd)). A similar result was obtained by Oliveira *et al.* (2014) when inoculating *P. australis* with an autochthonous microbial consortium resistant to copper (AMC(Cu)).

In this study the copper concentration in roots (and in rhizomes) of sediment co-contaminated with AMC(Cu) was 1.5 more than in treatment with sediment co-contaminated without AMC(Cu), and is 21.1 more, when compared with copper concentration present in roots of control treatment (Table 3). In addition, the presence of AMC(Cu) increased the difference between concentration

of copper present in roots and stems. This result showed that the inoculation of sediment co-contaminated with AMC(Cu) improved potential of phytostabilization.

Co-contaminated soil/sediment, are considered difficult to remediate due to the mixed nature of the contaminants and an alternative treatment to expensive conventional methods (excavation and incineration) can be bioaugmentation (Roane *et al.*, 2001). For instance, Weyens *et al.* (2010), after inoculation with endophytic bacteria also observed a significantly increased in nickel uptake by roots of the plant (*Lupinus luteus*) exposed to nickel and trichloroethylene (TCE).

The microorganisms associated to plant can improve phytoremediation of metal in co-contaminated sediment (Weyens *et al.*, 2010). However, studies applying bioaugmentation to co-contaminated soils/sediments are scarce (Roane *et al.*, 2001) and much less applied to saltmarsh sediments. Moreover, to our knowledge to date, it is nonexistent regarding the application of autochthonous bioaugmentation. This fact reinforces the importance of this work.

The bioavailability of metals/metalloids in soil depends on factors such as microbial activities. Several researchers have shown the positive impact of bioaugmentation on metal bioavailability, for instance, Braud *et al.* (2006), observed that when *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* are present the fraction exchangeable of lead increased 113%.

Oliveira *et al.* (2014) also observed that when AMC(Cu) was added to rhizosediment of *P. australis* the fraction of copper bioavailable in sediment increased resulting also in higher metal uptake by *P. australis*.

This is contrary to the observed in this present study, however in the present work a different plant species was tested.

In the present work, comparing several fractions of metal in sediment when petroleum was added to treatment with copper the percentage of metal in the fraction *exchangeable and bound to carbonates* decreased 5 points percentage (p.p.) and, in contrast, the percentage of metal in the fraction *bound to organic matter and sulphide and residual* increased in the same proportion (5p.p.).

For instance, Zhang *et al.*, (2009) observe no significant difference in the soil cadmium fractions between the treatment with only cadmium contamination and the treatments with co-contamination of cadmium and pyrene. This result indicated that interaction between metals/metalloids and organic pollutants no influence their adsorption onto soil particles. Zhang *et al.* (2012) propose that an interaction between metals/metalloids and organic pollutants may influencing their adsorption onto soil particles, however, was dependent on their concentration and properties, soil conditions such as pH and/or types of metals/metalloids and organic pollutants present in the soil/sediment co-contaminated.

The same trend is observed when AMC(Cu) was present together with copper and petroleum, the percentage of metal in the fraction *exchangeable and bound to carbonates* decreased 7p.p. and the percentage of metal in the fraction *bound to organic matter and sulphide and residual* increased 7p.p., when compared with *copper treatment* (Fig. 14).

Nunes da Silva *et al.* (2014) has reported for *J. maritimus* but for a different metal (cadmium) no differences between the percentages of the metal in each fraction with and without AMC(Cd). In contrast, in the same study, for *P. australis* the percentage of cadmium in the fraction *exchangeable and bound to carbonates* decreased, (i.e. the cadmium bioavailability was lower), when the sediment was inoculated with AMC(Cd).

The metabolism of the microorganisms associated to plant may indirectly and directly alter the uptake of metals/metalloids: indirectly by improving the growth of plants (via a variety of mechanisms including direct effects on nutrient availability and enhancement of root growth) and directly by altering the metal bioavailability in the rhizosphere (through acidification, chelation, precipitation, immobilization and oxidation-reduction reactions in the rhizosphere) (Ma *et al.*, 2011).

In fact, in this present study when the AMC(Cu) was present in treatment with both contaminants (copper plus petroleum) the metal uptake by the plant was higher than in treatments with the metal alone, despite the slightly lower

bioavailability of the metal. A possible explanation for this can be the presence of the organic pollutant (petroleum), as discussed in the previous section. In addition, when AMC(Cu) was present in the treatment with both contaminants the $EF_{\text{belowground}}$ was higher than in the same treatment without AMC(Cu) (Table 5), demonstrated that the addition of consortia microbial increased the phytostabilization potential of *J. maritimus*.

3. Conclusion

Inoculation of AMC(Cu) enhanced slightly the biomass of *J. maritimus* plants. Furthermore, inoculation promoted copper uptake and accumulation in roots, although not promoting the copper translocation to stems. So, the phytostabilization capacity of *J. maritimus* was enhanced. The presence of a second contaminant (petroleum) of a different chemical nature (organic) interfered with bioavailability of copper (decreasing it) in sediment but promoted *J. maritimus* uptake of the metal (increasing copper concentration), contributing for *J. maritimus* phytostabilization potential.

The role of soil/sediment microbiota (bacteria and fungi), specifically rhizospheric in the development of phytoremediation techniques has to be elucidated with the objective to speed up the process and to optimize the rate of mobilization/adsorption/accumulation of pollutants. To occur phytoremediation of metals/metalloids, is required the bioavailability of metals/metalloids in sediments. In this regard, it may be possible to employ autochthonous bioaugmentation to saltmarsh rhizosediment and to alter the bioavailability of metals for improving the potential phytoremediation of the metal contaminants on large scale in this environment.

CHAPTER IV

Effect of copper in petroleum hydrocarbons biodegradation by a saltmarsh plant (*Juncus maritimus*) - rhizosphere microorganisms association and the role of autochthonous bioaugmentation

1. Results

1.1.Plant biomass

1.2.Total Petroleum Hydrocarbons (TPHs) in sediment

1.3.Abundance of hydrocarbons degrading microorganisms

2. Discussion

2.1.Plants biomass and contaminants

2.2.Plants biomass and bioaugmentation

2.3.Effect of copper on petroleum bioremediation

2.4.Effect of bioaugmentation on petroleum biodegradation

3. Conclusion

Effect of copper in petroleum hydrocarbons biodegradation by a saltmarsh plant (*Juncus maritimus*) - rhizosphere microorganisms association and the role of autochthonous bioaugmentation

1. Results

Results of this investigation provide information regarding the effects of the presence of copper on petroleum hydrocarbons remediation potential of saltmarsh plants such as *J. maritimus* and their rhizospheric associated microorganisms, in a co-contaminated sediment. The potential of bioaugmentation was also assessed. For that, it was inoculated in the sediment three types of autochthonous microbial consortia previously prepared in laboratory (AMC resistant to copper, the AMC resistant to petroleum and the AMC resistant to copper plus petroleum). TPHs levels and MPNs in the sediments were measured. The experimental work for obtaining these results, featured in this chapter, is outlined in Chapter II (Materials and Methods).

1.1. Plant biomass

In the control treatment, the sediment was not doped with any of the contaminants, being exposed under the same experimental conditions as the other treatments. At the end of the experiment the biomass of each plant organ was obtained after drying to constant weight.

Biomass results in Table 7 were obtained by weighing, being expressed as means and standard deviation of three replicates for each treatment. For all values, the same statistical analysis, (*t*-student) was utilized.

Table 7 Dry weight (g) of the plant organs, (average and standard deviation, n=3) for each treatment (control; doped with petroleum (Pet); doped with Cu (Cu); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu

(Cu+Pet+AMC(Cu); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Pet); doped with Cu plus petroleum and AMC resistant to Cu plus petroleum (Cu+Pet+AMC(Cu+Pet)). Plant total weight per treatment is also included. Values showing different letters indicate significant differences in each plant organ ($p>0.05$, *t*-student).

Treatment	Biomass				
	Stems	Leaves	Rhizomes	Roots	Plant
Control	66±8 ^a	26±9 ^a	12±4 ^a	2,2±0,6 ^a	106±20 ^a
Cu	72±7 ^a	25±5 ^a	13±2 ^a	2,0±0,8 ^a	111±8 ^a
Pet	69±8 ^a	24±3 ^a	13±3 ^a	2,9±0,7 ^a	108±11 ^a
Cu + Pet	73±8 ^a	22±2 ^a	14±3 ^a	2,3±0,6 ^a	112±10 ^a
Cu + Pet + AMC(Cu)	83±12 ^a	29±4 ^a	14±2 ^a	2,5±0,2 ^a	128±17 ^a
Cu + Pet + AMC(Pet)	67±12 ^a	23±7 ^a	15±11 ^a	2±1 ^a	107±8 ^a
Cu + Pet + AMC(Cu+Pet)	60±12 ^a	18±6 ^a	13±2 ^a	3±1 ^a	94±16 ^a

Using as a reference the biomass of different organs of plants in *control* treatment (66g stems; 26g leaves; 12g rhizomes; 2.2g roots), no statistical differences ($p>0.05$) were observed when comparing these biomasses with the biomasses observed in the other treatments. However, in presence of the AMC(Cu+ Pet) there was a tendency for a decrease in total plant biomass, and in presence of the AMC(Cu) there was a tendency for an increase in total plant biomass. In both treatments these oscillations, decrease and increase, corresponded mainly to plant aboveground organs, stems in case of AMC (Cu) and stems plus leaves when AMC (Cu+Pet) was present.

When calculating the correlation of the biomass of each organ in the same treatment (Table 8), and comparing these values with the correlation values of another treatment, it can be seen that the relationship between the several treatments is, generally, maintained. Through the several treatments it appears that the roots biomass was on average 0.03 the biomass of the stems, 0.10 of the biomass of the leaves and 0.18 of the biomass of the rhizomes.

Table 8 Correlation of the average biomass between several organs of the *J. maritimus*.

Treatment	Correlation of the biomass			
	Stems	Leaves	Rhizomes	Roots
Control	66	26	12	2.2
	0.39x →			
	0.46x →			
	0.18x →			
	0.08x →			
	0.03x →			
Pet	69	24	13	2.9
	0.35x →			
	0.54x →			
	0.22x →			
	0.12x →			
	0.04x →			
Cu	72	25	13	2.0
	0.35x →			
	0.52x →			
	0.15x →			
	0.08x →			
	0.03x →			
Cu + Pet	73	22	14	2.3
	0.30x →			
	0.64x →			
	0.16x →			
	0.10x →			
	0.03x →			
Cu + Pet + AMC(Cu)	83	29	14	2.5
	0.35x →			
	0.48x →			
	0.18x →			
	0.08x →			
	0.03x →			
Cu + Pet + AMC(Pet)	67	23	15	2.3
	0.34x →			
	0.65x →			
	0.15x →			
	0.10x →			
	0.03x →			
Cu + Pet + AMC(Cu+Pet)	60	18	13	2.7
	0.30x →			
	0.72x →			
	0.20x →			
	0.15x →			
	0.04x →			

In Fig. 15 are displayed the percentages of the biomass of each organ relative to the total weight of *J. maritimus*. In *J. maritimus* about 85% of plant weight was localized at aboveground, wherein more than 60% were related to stems. About 15% of plant weight was localized belowground.

Taking into account all treatments, including the control, a low range of variation in the biomass weight percentages was observed for rhizomes [11-14%], leaves [19-25%] and stems [62-65%], being the roots the plant organ with less weight percentage variation [2-3%] (Fig. 15).

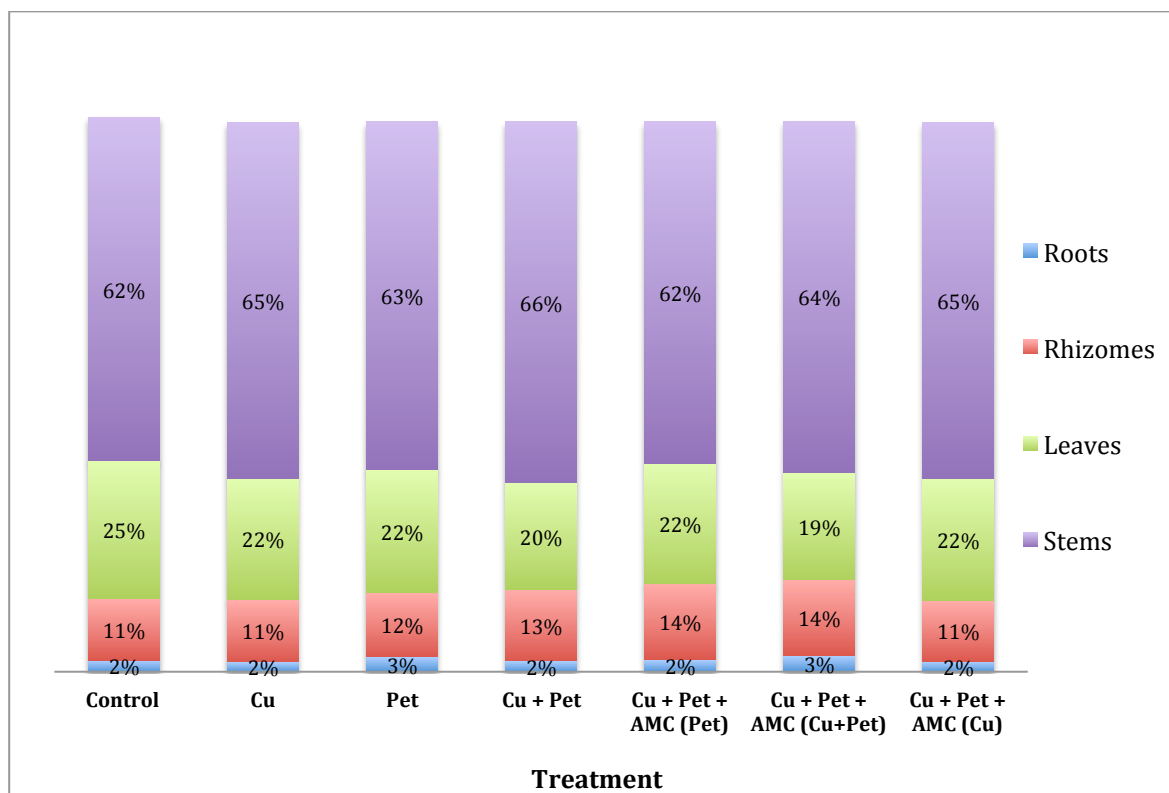


Figure 15 Percentage (%) of each plant organ biomass weight (roots; rhizomes; leaves and stems) relatively to the total biomass of *J. maritimus* in the several treatments.

1.2.Total Petroleum Hydrocarbons (TPH) in sediments

In the present work, the concentration of TPHs in doped sediments ranged from 2.12 mg/g_{dry sediment} to 2.9 mg/g_{dry sediment} (Fig. 16). Significantly ($p < 0.05$) lower TPHs concentrations in sediments at time T5 (five month after) than in time T0 (when the sediment was contaminated) were observed for all treatments.

Five months after contaminating the sediment with petroleum, 39% of TPHs initial concentration (2.9 mg/g_{dry sediment}) was degraded. But when petroleum was added together with copper (treatment (Cu+Pet)) the percentage of TPHs degradation was only 25% (Table 9), i.e., 14 points percentage (p.p.) less. The presence of copper affected the degradation of petroleum hydrocarbons, the differences in TPHs being statistically significant ($p < 0.05$) between treatment with *petroleum only* and treatment with *copper plus petroleum* (Fig. 16).

When petroleum was added together with copper and AMC(Pet) the percentage of TPHs degradation was 22% (Table 9). The differences were not statistically significant ($p>0.05$) among treatments with *copper plus petroleum* and treatment with *copper plus petroleum and AMC(Pet)* (Fig. 16). This results indicates that the presence of autochthonous microbial consortium resistant to petroleum AMC(Pet) did not increased the degradation of petroleum hydrocarbons.

When petroleum was added together with copper and AMC(Cu+Pet) the percentage of TPHs degradation was only 21% (Table 9). The differences in TPHs were not statistically significant ($p>0.05$) when comparing the treatment with *copper plus petroleum and AMC(Cu+Pet)* and treatment with *copper plus petroleum* (Fig. 16). This results indicates that the presence of autochthonous microbial consortium resistant to petroleum and resistant to copper (AMC(Cu+Pet)) did not promoted hydrocarbons degradation.

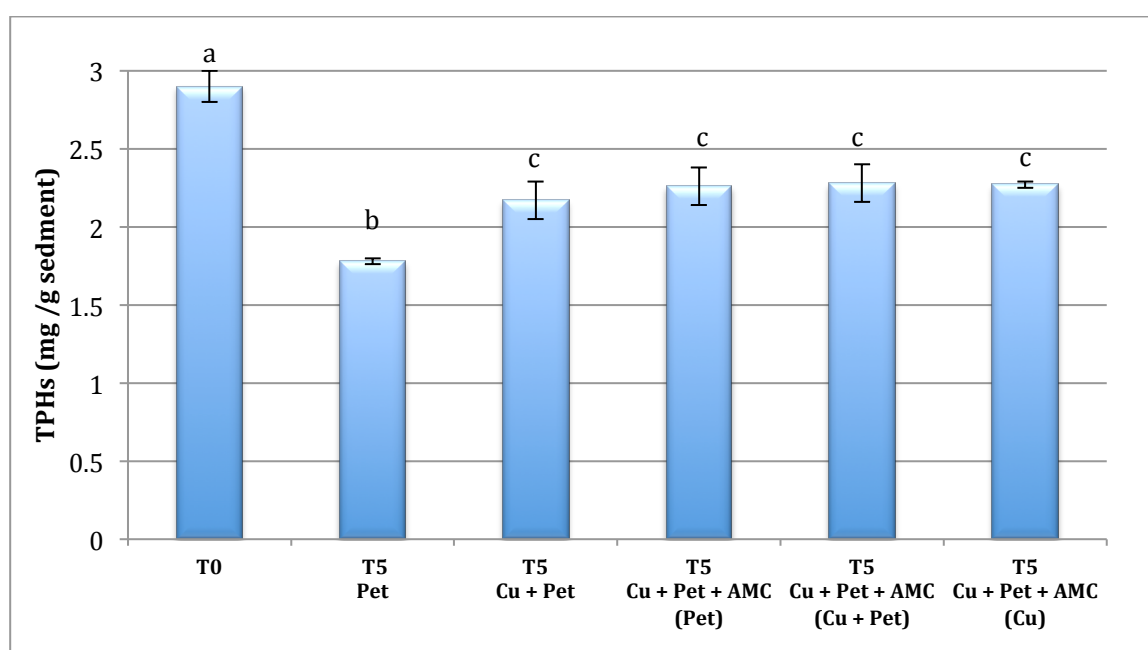


Figure 16 TPHs concentration in sediment (mg/g_{sediment}) at initial time (T0) and in different treatments after five month (T5). Columns with different letters are significantly different ($p<0.05$, *t*-student) and with the same letters indicate differences not statistically significantly ($p>0.05$, *t*-student). Error bar show standard deviation ($n=3$).

When petroleum was added together with copper and AMC(Cu) the percentage of TPHs degradation was 22% (Table 9). The differences in TPHs were not statistically significant ($p>0.05$) when comparing the treatment with *copper*

plus petroleum and AMC(Cu) and treatment with *copper plus petroleum* (Fig. 16). This results shows that the presence of autochthonous microbial consortium resistant to copper AMC(Cu) did not interfere in the degradation of petroleum hydrocarbons.

Table 9 TPHs degradation rates (%) in sediment five months (T5) after contamination of the sediment, in the different treatments: doped with petroleum (Pet); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)); doped with Cu plus petroleum and AMC resistant to petroleum (Cu+Pet+AMC(Pet)); doped with Cu plus petroleum and AMC resistant to Cu plus petroleum (Cu+Pet+AMC(Cu+Pet)).

Treatment	TPHs degradation rate (%)
	Sediment
Pet	39%*
Cu+Pet	25%*
Cu + Pet + AMC(Cu)	22%*
Cu + Pet+AMC(Pet)	22%*
Cu + Pet + AMC(Cu+Pet)	21%*

*These values were obtained using the equation $(C_0 - C)/C_0 \times 100$; (C_0 is average of the TPHs concentration value ($2.9 \text{ mg/g}_{\text{dry sediment}}$) when the sediment was contaminated (T0) and C is the average TPHs concentration value ($\text{mg/g}_{\text{dry sediment}}$) five month after (T5) in each treatment).

1.3. Abundance of hydrocarbons degrading microorganisms

Most probable number (MPN) method was selected to estimate the number of hydrocarbon-degraders (HD) microorganisms present in the samples of the sediments immediately after sample collection. The data present in Fig. 17 is logarithmic values of the average of HD microorganisms present for gram of sediments in each treatment. In the present work, after 5 months, these values ranged from $5.71/\text{g}_{\text{dry sediment}}$ to $9.43/\text{g}_{\text{dry sediment}}$ (Fig.17).

In the treatment with *copper* the abundance of HD microorganisms decreased significantly ($p < 0.05$) (31%) when compared with *control* treatment.

When petroleum was present, alone or combined with copper, no significant differences ($p>0.05$) were observed in abundance of HD microorganisms, when compared with *control* treatment. The same was observed, when comparing treatment with *copper plus petroleum and AMC(Pet)* and *control* treatment (Fig. 17).

Differences were statistically significant ($p<0.05$) between *petroleum* treatment and *copper* treatment, in the *petroleum* treatment the abundance of HD microorganisms being 25% more than in *copper* treatment. The same trend was observed when compared treatment with *copper plus petroleum* and *copper* treatment, the abundance of HD microorganisms was more 28% in *copper plus petroleum* than in *copper* treatment.

In addition, the same trend was observed when comparing treatment with *copper plus petroleum and AMC(Pet)* with *copper* treatment, the abundance of HD microorganisms being more 29% in *copper plus petroleum and AMC(Pet)* than in *copper* treatment, and the differences were statistically significant ($p<0.05$) between these treatments.

In the presence of AMC (Cu+Pet) the abundance of HD microorganisms decreased significantly ($p<0.05$) (38%) when compared with *control* treatment. The same trend in abundance of HD microorganisms was observed, when the AMC (Cu) was added together with *copper and petroleum* and compared with abundance of HD microorganisms in *control* treatment (Table 10). The abundance of HD microorganisms in *copper plus petroleum and AMC(Cu)* treatment was less 39% than in *control* treatment, the abundance HD microorganisms in sediment being significantly lower ($p<0.05$) when compared with *control* treatment (Fig.17).

In the presence of AMC(Pet) the abundance of HD microorganisms was more 31% than when AMC(Cu+Pet) was added, the abundance HD microorganisms in this treatment was significantly higher ($p<0.05$) relatively to *copper plus petroleum and AMC(Cu+Pet)* treatment.

The same trend in abundance of HD microorganisms was observed, when compared *copper plus petroleum and AMC(Pet)* treatment with *copper plus*

petroleum and AMC(Cu) treatment: the abundance of HD microorganisms was more 32% in the presence of AMC(Pet) than when in the presence of AMC(Cu), the abundance HD microorganisms in this treatment with AMC(Pet) was significantly higher ($p<0.05$) relatively to treatment with *copper plus petroleum and AMC(Cu)* (Fig. 17).

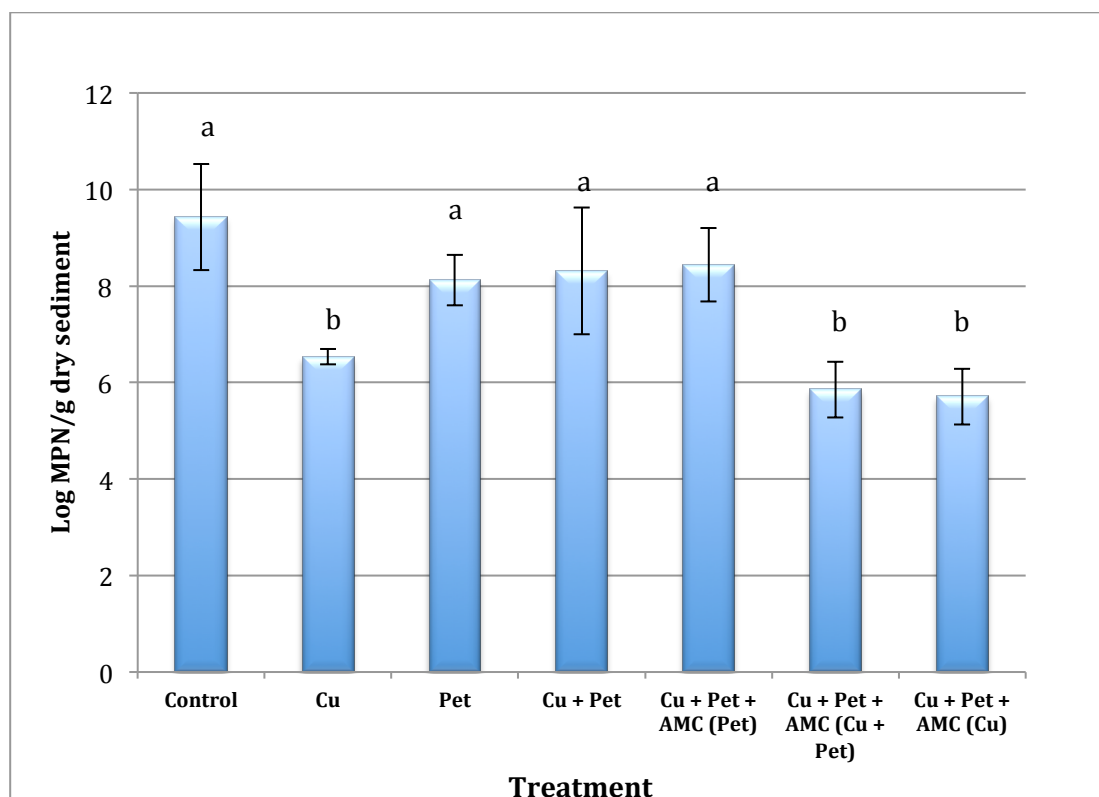


Figure 17 The number of hydrocarbons degraders microorganisms (average (Log MPN/g dry sediment) and standard deviation, $n=3$) in several treatment (control; doped with Cu (Cu); doped with petroleum (Pet); doped with Cu plus petroleum (Cu+Pet); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu)); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Pet)); doped with Cu plus petroleum and AMC resistant to Cu (Cu+Pet+AMC(Cu+Pet)). Columns are showing different letters indicate statistically significant differences ($p<0.05$, t -student) and the same letters indicate differences not statistically significantly ($p>0.05$, t -student).

Table 10 Correlation between treatments in the numbers of the HD microorganisms present in sediment and calculated by MPNs protocol. The (=) sign is an indication of not significantly different ($p>0.05$) between treatments (#) and (##) in the numbers of the HD

microorganisms present in sediment. The (+) sign is an indication of increasing the (-) sign indicates to decrease.

Treatment		MPNs Sediment
Cu [#]	Control ^{##}	(-)31%*
Pet [#]	Control ^{##}	=
	Cu ^{##}	(+)25%*
Cu+Pet [#]	Control ^{##}	=
	Cu ^{##}	(+)28%*
	Pet ^{##}	=
Cu + Pet+AMC(Pet) [#]	Control ^{##}	=
	Cu ^{##}	(+)29%*
	Pet ^{##}	=
	Cu+Pet ^{##}	=
Cu + Pet + AMC(Cu+Pet) [#]	Control ^{##}	(-)38%*
	Cu ^{##}	=
	Pet ^{##}	(-)28%*
	Cu+Pet ^{##}	(-)30%*
	Cu + Pet+AMC(Pet) ^{##}	(-)31%*
Cu + Pet + AMC(Cu) [#]	Control ^{##}	(-)39%*
	Cu ^{##}	=
	Pet ^{##}	(-)30%*
	Cu+Pet ^{##}	(-)31%*
	Cu + Pet+AMC(Pet) ^{##}	(-)32%*
	Cu + Pet + AMC(Cu+Pet) ^{##}	=

* These values were obtained using the equation $((y-x)/y) \times 100$; (x is the treatment is indicated for (#) and y is the treatment indicated for (##)).

2. Discussion

The feasibility of phytoremediation for co-contaminated soils/sediments has recently been investigated (Singer *et al.*, 2007; Lin *et al.*, 2006, 2008; Zhang *et al.*, 2009; Rengel *et al.*, 2011; Sun *et al.*, 2011; Weyens *et al.*, 2011; Li and Wong, 2012; Wang *et al.*, 2012; Hechmi *et al.*, 2015). However, studies involving wetlands plants are scarce (Rengel *et al.*, 2011; Heichmi *et al.*, 2015) and more research is needed so that it can be applied for instance to estuarine areas. Remediation of soils/sediments co-contamination (organic and metals/metalloids) is a complex process, because metals/metalloids and organic may interact among themselves (Zhang *et al.*, 2011) and interfere with plant growth, soil/sediments processes and rhizosphere biota (Almeida *et al.*, 2008).

Studies on the effect of metals/metalloids on organic pollutant biodegradation, demonstrate that metals/metalloids have the potential to inhibit pollutant biodegradation (e.g. Amor *et al.*, 2001; Alisi *et al.*, 2009) under aerobic and anaerobic condition (Sandrin and Maier, 2003).

The present study was designed to investigate the influence of copper in petroleum hydrocarbons to biodegradation by a saltmarsh plant (*Juncus maritimus*) in association with its rhizosphere microorganisms and to investigate the possible positive role of autochthonous bioaugmentation.

2.1. Plant biomass and contaminants

It is well recognized that oil spills may affect plants biomass negatively, by decreasing plant height, stem density and biomass or causing complete mortality (e.g. Lin and Mendelssohn 2009). On the other hand, metals/metalloids can also affect plants biomass, however, in co-contamination situation, the results can be different.

Khan *et al.* (2009) considered that the interaction between organic pollutant and metal/metalloids can either cause a negative or positive effect on plant biomass depending on the type and concentration of both pollutants.

Lin *et al.* (2008) observed that copper decrease shoot dry weight, but in the presence of pyrene co-contamination the yields of shoot tended to increase, suggesting that certain concentrations (50-100-500 mg/kg) of pyrene can alleviate the inhibition of copper to *Zea mays* L.. Zhang *et al.* (2012) reports a significant increase in total biomass of *Juncus subsecundus* in cadmium treatment with PAHs when compared to the cadmium treatment without PAHs. On the other hand, Wang *et al.* (2012) observed that the presence of PAHs decreased the stimulating effect of cadmium on the growth of the hyperaccumulator plant (*S. alfredii*). Chigbo and Batty (2015), observed that the presence of copper in the soil did not affect the growth of *Zea mays*, while pyrene and a combination of both significantly decreased the growth of *Z. mays* after 60 days of planting. The interaction between organic and metals/metalloids may exacerbate the phytotoxicity of the contaminants and thus resulting in a decrease in biomass weight (Wang *et al.*, 2012).

In the present study, results indicating that there was not a significant effect of co-contamination in *J. maritimus* biomass, the differences between total plant biomass of *J. maritimus* not being statistically significant ($p>0.05$) when comparing treatments with *petroleum* and with *petroleum plus copper*. No significant differences ($p>0.05$) were also observed among the biomass of the different structures (roots, rhizomes or shoots) when comparing treatments with *petroleum* and with *petroleum plus copper* (Table 7).

These results suggest that interactions of copper and petroleum did not have antagonistic and synergistic effects on the biomass weight of the different structures of *J. maritimus*. Plants growth, in special of the roots, is important for organic compound biodegradation, because, root growth can promote soil aeration, which could enhance oxidative degradation of organic compounds, and this may also widen the way for trapped contaminants to become accessible to degrading bacteria (Leigh *et al.*, 2002).

2.2. Plant biomass and bioaugmentation

Microbial inoculations in polluted soils/sediments can help the plant through toxin decomposition as well as promoting plant growth.

After five month, no statistically significant differences were observed either in biomass of several organ (root; rhizomes; stems) nor in total biomass of *J. maritimus* when inoculation either AMC (AMC(Cu); AMC(Pet); AMC(Cu+Pet) in sediment co-contaminated. However, in presence of the AMC(Cu+ Pet) there was a tendency for a decrease in total plant biomass, and in presence of the AMC(Cu) there was a tendency for an increase in total plant biomass, indicating a possible effect of bioaugmentation on this parameter. For instance, Li and Wong (2012) reported a reduction in plant biomass of *Sedum alfredii* with the inoculation of bacteria (*Burkholderia cepacia*), in sediment co-contaminated. In contrast, Weyens *et al.* (2010), reported a significantly increased in root of *Yellow lupine* when inoculated with *Burkholderia cepacia* in co-contaminated water.

2.3. Effect of copper on petroleum biodegradation

Remediation refers to removing, transforming or degrading contaminants to harmless or less harmful substances (Jain *et al.*, 2011). The mechanisms underlying dissipation/remediation of organic pollutants in soils/sediments are biodegradation, photodegradation, volatilization, plant uptake and metabolism and incorporation into soil organic material (Ma *et al.*, 2011).

In general, plants can promote dissipation/remediation of organic pollutants by immobilization, removal and/or promotion of microbial degradation (Megharaj *et al.*, 2011). On the other hand, the support of the growth and metabolic activities of microbial communities capable of degrading organic pollutants in the rhizosphere is based on the secretion by plants of root exudates, as these root exudates (i.e., phenolics, organic acids, alcohols, proteins) may serve as carbon and nitrogen sources to microorganisms (Alkorta and Garbisu, 2001). Plants also release a number of enzymes (laccases, dehalogenases, nitroreductases, nitrolases and peroxidases) into soils/sediments and these enzymes can degrade organic contaminants (Alkorta and Garbisu, 2001). In the case of the microbial degradation of petroleum hydrocarbons it can be mediated by specific enzymes systems, Cytochrome P450, alkane, hydroxylases, consist in a super family of ubiquitous *Heme-thiolate Monooxygenases* with an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives and other compounds (Van Beilen and Funhoff, 2007; Das and Chandran, 2011).

In this study, after the experimental time (five months) it was observed remediation/degradation of petroleum hydrocarbons, with a decrease of 39% in their level relative to initial levels (measured as TPH after doping with petroleum) (Fig. 16). These results indicated that the microbial communities present in the rhizosphere of *J. maritimus* had potential to degrade petroleum hydrocarbons. In fact, results obtained by Ribeiro *et al.* (2011) demonstrated that different saltmarsh plants (*J. maritimus*; *P. australis*; *T. striata*) have capabilities to retain hydrocarbons around their roots, and for fostering hydrocarbons degrading microorganisms in its rhizosphere which can result in hydrocarbons degradation. Diverse studies have demonstrated the importance of the *rhizosphere effect* on

the degradation of hydrocarbons, for instance, Wang *et al.* (2008) concluded that the presence of petroleum pollutants and plant rhizosphere promoted the increase of microorganisms that could degrade soil petroleum hydrocarbons.

Metal/metalloids ions are able to form unspecific complexes with different compounds within microbial cells, resulting in toxic effects that inhibit microbial activities (Roane *et al.*, 2001). In this sense, co-contamination may affect the biodegradation degree of organic compounds (Amor *et al.*, 2001; El-Deeb and Altalhi, 2009). In this study, when copper was added to sediment with petroleum, there was a decrease in TPHs degradation rates (from 39% to 25%) (Fig. 16). Moreover, the abundance of HD microorganisms also decreased in the presence of copper. This result indicates copper had some inhibitory effect on petroleum hydrocarbons biodegradation and/or in HD microorganisms abundance.

This effect was similar with other studies carried out in medium co-contaminated. Almeida *et al.* (2013a) reported, that the presence of copper in sediment had some deleterious effect on the hydrocarbons degradation process because the results showed a decreased in hydrocarbons degradation rate and/or in the HD microorganisms abundance. In contrast, Baldrian *et al.* (2000) observed no inhibitory effect on PAHs degradation in soil containing a high concentration of Cd.

Several studies have demonstrated that biodegradation of organic pollutants can be diminished by metals/metalloids toxicity (Said and Lewis, 1991; Sandrin *et al.*, 2000; Sandrin and Maier, 2003). For instance, studies of metal influence on microbial biodegradation of aromatic and aliphatic hydrocarbons have demonstrated biodegradation inhibition caused by microbial activity inhibition due to metal toxicity namely of Cd, Cr, Cu, Hg, Ni, Pb and Zn (Amor *et al.*, 2001; Riis *et al.*, 2002). Metals/metalloids appear to interfere with both the physiology and ecology of organic degrading microorganisms (Sandrin and Maier, 2003).

The presence of metals/metalloids in soil/sediment may inhibit organic pollutant biodegradation through interaction with enzymes directly involved in biodegradation or through interaction with enzymes involved in general metabolism (Sandrin and Maier, 2003). Toxic effects of metal/metalloids may be exerted through different mechanisms, for instance, as substitution within an

enzyme of physiologically essential ions by toxic metal ions, thus rendering the enzyme non-functional (Nies, 1999), and imposition of oxidative stress on microorganisms (Kachur *et al.*, 1998). On the other hand, metal ions are able to form unspecific complexes with different compounds within microbial cells, that resulting in toxic effect that inhibits microbial activities (Roane *et al.*, 2001).

The form/bioavailability in which the metals/metalloids is present in the soils/sediments can be extremely important in biodegradation of organic pollutant. Hoffman *et al.* (2005) and Olaniran *et al.* (2011) reported that the type of medium determines the degree and patterns by which metals/metalloids inhibit biodegradation of organic pollutants. This is partly due to the fact that metals can exist in a range of different physical and chemical forms that are affected by environmental conditions, that include pH and ionic strength of the water phase, as well as soil/sediment properties such as: grain size, clay type and content, organic matter content and ion exchange capacity of the solid phase (Sandrin and Maier, 2003). Therefore, reporting bioavailable metal concentration represents a key step in standardizing methods to quantify metals/metalloids impacts on biodegradation (Olaniran *et al.*, 2011).

As discussed in chapter III, in this study the metal became less bioavailability in the presence of petroleum, which could reduce copper toxicity to microorganisms. In fact, HD microorganisms' abundance was higher when copper was mixed with petroleum than when copper was alone.

On the other hand, the soil organic matter plays an important role in the fate and transport of many organic contaminants, hence plays a role in hydrocarbon degradation. Hydrocarbons tend to partition to soil organic matter, and once sorbed, their degradation may be dependent on the rate at which these chemicals desorb into the aqueous phase (Karthikeyan and Kulakow, 2003). In fact, sediment with small grain (low density fraction) and high organic content may be the main factors that limit hydrocarbons bioavailability, which reduces the rate and extent of degradation (Young *et al.*, 2002). Almeida *et al.* (2013a) considers that the microbial degradation potential and metal/metalloids influence varied with soil/sediment characteristic probably resulting from differences in contaminant bioavailability.

In this present study, the sediment used has a low organic matter percentage (ca. 3%, Ribeiro *et al.*, 2013a), and therefore should not be a constraint factor for hydrocarbons bioavailability.

2.4. Effect of bioaugmentation on petroleum biodegradation

The approach assumed in this part of the study was based on performing bioaugmentation, with three different microbial consortia: one autochthonous microbial consortium resistant to copper that could protect the system plant-microorganisms of copper toxicity (AMC(Cu)); a second autochthonous microbial consortium resistant to petroleum toxicity and with potential to degrade this organic compound (AMC(Pet)); and a third consortia resistant to copper and petroleum with potential to degrade this organic compound and resistant to both contaminants (AMC(Cu+Pet)).

Microbial breakdown of hydrocarbon pollutants is generally a very slow process, but bioremediation/biodegradation could be improved if the right environmental conditions such as: pH, temperature, nutrients and relevant microbial consortia are present (Akpoveta *et al.*, 2011). The characterization of microorganism strains petroleum-degrading and their metabolic pathways serves to improve bioremediation/biodegradation approaches (Peixoto *et al.*, 2011).

Bioaugmentation offers a way to provide specific microbes in sufficient number to optimize the biodegradation (Alisi *et al.*, 2009). There are many reports on addition of microbial strain resistant to the pollutant to enhance its degradation for treatment of soils/sediments containing organic/inorganic contaminants (Roane *et al.*, 2001; Sandrin and Maier, 2003; Weyens *et al.*, 2010; Atagana *et al.*, 2011; Gutiérrez-Guínés *et al.*, 2014) but they are scarce reports of bioaugmentation with microbial consortia in this situation of co-contamination.

In the case of co-contamination, different microbial consortia can be tested. For instance, it can be promoted the number of microorganisms with capacity to resist and/or degrade the organic pollutant.

The approaches to mitigate the extent to which metals/metalloids inhibit organic biodegradation have involved lowering metal/metalloids bioavailability and/or increasing metal/metalloids resistance to facilitate biodegradation (Sandrin and Hoffman, 2007). Inoculation with metal resistant microorganisms that can increase metals/metalloids resistance can also be an option to facilitate biodegradation in co-contamination situation (Sandrin and Hoffman, 2007).

Another option is a microbial consortium resistant to both contaminants.

The data of MPN (Fig.17) obtained in this study, shows that the consortia did not increase petroleum-degraders microorganisms, because, the number of MPN was the same with or without AMC(Pet). In fact, the degradation rate of petroleum hydrocarbons was identical with or without AMC(Pet). Several studies have demonstrated that (under certain conditions), inoculation accelerates the rates and the degree of diesel oil biodegradation but that was not the case in the present study.

In a situation of co-contamination, the presence of any type of the AMC (alone or combined) resulted in similar percentage of petroleum degradation. The inoculation of any of the microbial consortia did not increase the percentage of degradation of petroleum hydrocarbons in co-contamination situation. Also, the inoculation of any type of the AMC (alone or combined) did not increase the number of HD microorganisms in the sediment when compared with treatment with the same contamination (copper plus petroleum) but without AMC. In fact, when inoculated with AMC(Cu) or with AMC(Cu+Pet) the number of HD was significantly ($p < 0.05$) lower, when compared with AMC(Pet) (Fig.17). Therefore, the presence of microorganisms resistant to copper may have had some interference in the composition of the bacterial consortium. Doelman *et al.* (1994) speculated that in soils contaminated with metal the resistances to this may reduce the bacterial bioremediation capability towards chlorinated aromatics and polyaromatic hydrocarbons (PAHs), although others authors have proven the capacity of metal-resistant strains to play a role in improving bioremediation of organic pollutants (Roane *et al.*, 2001). However, in these studies no plants were present.

So, present results indicate that in the present conditions bioaugmentation did not promote the bioremediation potential of the *J. maritimus* associated rhizospheric microorganisms.

3. Conclusion

The results obtained in this part of the study suggest that copper and petroleum (alone or combined) did not cause toxic effects to *J. maritimus* and the rhizosphere of this saltmarsh plant have microorganisms with potential to petroleum degradation. However, the presence of copper interfered with this process of biodegradation, reducing the potential of the association *J. maritimus*-rhizospheric microorganisms for bioremediation of petroleum hydrocarbons in a co-contaminated (copper plus petroleum) sediment.

The inoculation of any type of the AMC did not lead to an increase in the number of HD microorganisms, consequently, it did not played a significant role in rhizodegradation of petroleum hydrocarbons. So, bioaugmentation was not effectively to overcome the negative effect of the metal on petroleum hydrocarbons degradation in a situation of co-contamination.

CHAPTER V

Conclusions and future directions

Conclusions and future directions

Phytoremediation is a promising technology for the treatment of sites contaminated with pollutants of different nature (organic and inorganic). This study elaborated on the potential of using *J. maritimus* and the microbial community present in its rhizosphere for the remediation of saltmarsh sediment contaminated with both copper and petroleum hydrocarbons. Pollutants were tested alone or combined.

The presence of copper and petroleum hydrocarbons, alone or in a mixture, did not affect, in general, *J. maritimus* biomass weight. In addition, when AMC(Cu) was added there was a tendency for a higher plant biomass, although the differences were not significant. *J. maritimus* seem to have the potential to remediate copper in contaminated and co-contaminated saltmarsh sediment, being able to deal with any toxicity that copper might impose.

As expected copper was mainly accumulated in roots of *J. maritimus* and a small quantity was translocate to stems. The presence of petroleum together with copper in rhizosediment did not significantly influence the copper translocation (i.e. the levels of copper in stems did not increase) or the copper accumulation into rhizomes. In contrast, the uptake of copper in roots was significantly increased in the presence of petroleum and copper together, despite the fact that the metal bioavailability was lower. These differences in the distribution of copper in the different plant tissues can result of different allocation strategies used by *J. maritimus*, such as restriction of upward movement of metal into stems and sub-cellular compartmentalization of metals, which allow them to prevent metal toxicity and cope with metal stress.

The presence of petroleum could mitigate the toxicity of the metal to the plants, leading to an increase of copper levels in plant. However, metal translocation did not increase, indicating that petroleum can have been complexed with copper and bound to the layers of lipophilic root. On the other hand, the presence of petroleum might have interfered in exudation of copper ligands released by roots and capable of forming soluble and bioavailable copper complexes. This result may suggest the interaction of copper with petroleum could affect negatively metal bioavailability in rhizosediments, however,

potentiated the phytostabilization capacity of *J. maritimus*. The same effect in metal bioavailability was observed when AMC(Cu) was present, with the application of bioaugmentation technique in a co-contamination situation increasing 1.5 times the stabilizing potential of the *J. maritimus*. This seems to indicate that the addition of the AMC(Cu) inoculum promoted the stabilization capacity of *J. maritimus*.

The microbial community present in rhizosediment of *J. maritimus* had potential to degrade petroleum hydrocarbons. The addition of petroleum to rhizosediment did not change the number of petroleum hydrocarbons degraders microorganisms. But, the addition of copper had a toxic effect in these microorganisms and consequently on the percentage of petroleum hydrocarbons degraders, decreasing it. In addition the metal became less bioavailable in the presence of petroleum.

The inoculation of any type of the autochthonous microbial consortia (AMC(Cu); AMC(Pet); AMC(Cu+Pet)), did not lead to an increase in the abundance of HD microorganisms, consequently, it did not play an important role in rhizodegradation of petroleum hydrocarbons. So, the introduction of a microbial consortium with pollutant-resistance microorganisms did not result in a viable strategy to mitigate metal-inhibition of petroleum hydrocarbons biodegradation.

Future directions

This study elaborated on the potential of using saltmarsh autochthonous plants (*J. maritimus*) and respective associated microorganisms in phytoremediation of saltmarsh sediment contaminated with both copper and petroleum. *J. maritimus* could mitigate the toxicity effect of pollutants in the saltmarsh sediments by adsorption/absorption of the metal in the roots (phytostabilization) and degrading the organic pollutants (rhizoremediation). Plant – microbial community combinations could offer a promising approach towards phytoremediation of contaminated saltmarsh sediments. In addition, the application of bioaugmentation technique proved to be a promising technique to enhance metal remediation in co-contamination sediments of saltmarsh, but the same did not

occur in the case of petroleum hydrocarbons remediation (biodegradation), a pollutant of different nature.

Future studies are needed in order to address questions raised in this study.

Firstly: for what reason bioaugmentation did not function with HD microorganisms? A more detailed study of microbial community structure needs to be done in the future to explore the specific groups that are responsible for what occurred in the treatment with both copper and petroleum.

Secondly: in what sense the results would be different, if this experimental design it was done with other salt marsh plants, for instance, *P. australis*, which has different anatomical features.

References

- Aafi**, N.E., Brhada, F., Dary, M., Maltouf, A.F., Pajuelo, E., 2012. Rhizostabilization of metals in soils using *Lupinus luteus* inoculated with the metal resistant rhizobacterium *Serratia* sp., MSMC 541. *International Journal Phytoremediation*, 14(3): 261–274.
- Abdel-Ghani**, N.T., Hefny, M., El-Chagbaby, G.A.F., 2007. Removal of lead from aqueous solution using low cost abundantly available adsorbents. *International Journal of Environmental Science and Tecnology*, 4(1): 67- 73.
- Abhilash**, P.C., Jamil, S., Singh, N., 2009. Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. *Biotechnology Advance*, 27(4):474-488.
- Agnello**, A.C., Bagard, M., van Hullebusch, E.D., Esposito, G., Huguenot, D., 2015. Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation – assisted phytoremediation. *Science of the Total Environmental* (in press)
- Alford**, E.R., Pilon-Smits, E.A.H., Paschke, M.W., 2010. Metallophytes – a view from the rhizosphere. *Plant and Soil*, 337(1-2): 33–50.
- Ali**, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals - Concepts and applications. *Chemosphere*, 91(7):869-881.
- Alisi**, C., Musella, R., Tasso, F., Ubaldi, C., Manzo, S., 2009. Bioremediation of diesel oil a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. *Science of the Total Environment*, 407(8): 3024-3032.
- Alkio**, M., Tabuchi, T.M., Wang, X., Colon-Carmona, A., 2005. Stress response to polycyclic aromatic hydrocarbons in *Arabidopsis* include growth inhibition and hypersensitive response-like symptoms. *Journal of Experimental Botanic*, 56(421): 2983-2994.
- Alkorta**, I., and Garbisu, C., 2001. Phytoremediation of organic contaminants in soils *Bioresource Technology*, 79(3):273-276.
- Alkorta**, I., Hernandez-Allica, J., Becerril, J.M., Amezcaga, I., Albizu, I., Garbisu, C., 2004. Recent findings on the phytoremedation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. *Reviews in Environmental Science and BioTechnology*, 3(1): 71–90.
- Akpoveta**, O.V., Egharevba, F., Medjor, O.W., 2011. A pilot study on the biodegradation of hydrocarbon and its kinetic on kerosene simulated soil. *International Journal of Environmental Science*, 2(1):54-67.
- Almeida**, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2004. Influence of the sea rush *Juncus maritimus* on metal concentration and speciation in estuarine sediment colonized by the plant. *Environmental Science and Technology*, 38(11): 3112-3118.
- Almeida**, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2006a. Comparison of the role of the sea club-rush *Scirpus maritimus* and the sea rush *Juncus maritimus* in terms of concentration speciation and bioaccumulation of the metals in the estuarine sediments. *Environmental Pollution* 142(1):151-159.
- Almeida**, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2006b. Variability of metal contents in the sea rush *Juncus maritimus* - estuarine sediment system through one year of plant's life. *Marine Environmental Research*, 61(4): 424-438.

Almeida, C.M.R., Mucha, A.P., Delgado, M.F.C., Caçador, M., Bordalo, A.A., Vasconcelos, M.T.S.D., 2008. Can PAHs influence Cu accumulation by salt marsh plants? *Marine Environmental Research*, 66(3): 311–318.

Almeida, C.M.R., Dias, A.C., Mucha, A.P., Bordalo, A.A., Vasconcelos, M.T.S.D., 2009. Study of the influence of different organic pollutants on Cu accumulation by *Halimione portulacoides*. *Estuarine Coastal and Shelf Science*, 85(4): 627– 632.

Almeida, C.M.R., Mucha, A.P., Vasconcelos, M.T., 2011. Role of different salt marsh plants on metal retention in an urban estuary (Lima estuary, NW Portugal). *Estuarine Coastal and Shelf Science*, 91(2):243-249.

Almeida, R., Mucha, A.P., Teixeira, C., Bordalo, A.A., Almeida, C.M.R., 2013a. Bioremediation of petroleum hydrocarbons in estuarine sediments: Metal influence. *Biodegradation* 24(1): 111-123.

Almeida, C.M.R., Rocha, A.C., Mucha, A.P., Vasconcelos, M.T.S.D., 2013b. Evaluation of the Potential of Salt Marsh Plants for Metal Phytoremediation. *In: Heavy Metal Stress in Plants* Gupta D.G., Corpas, F.J., Palma, J.M., (Eds.). Springer, Verlag, Berlin, Heidelberg, 225-239.

AL-Saleh, E. S., and Obuekwe, C. 2005. Inhibition of hydrocarbon bioremediation by lead in a crude oil contaminated soil. *International Biodeterioration and Biodegradation*, 56(1): 1–7.

Amor, L., Kennes, C., Veiga, M.C., 2001. Kinetics of inhibition in the biodegradation of monoaromatic hydrocarbons in presence of heavy metals. *Bioresource Technology*, 78(2):181-185.

Anoliefo, G. O., Vwioko, D. E., Mpamah, P., 2003. Regeneration of *Chromolaena odorata* (L.) K. & R. in crude oil polluted soil: A possible phytoremediating agent. *Benin. Science Digest*, 1(1):9-16.

Arthur, E.L., Rice, P.J., Rice, P.J., Anderson, T.A., Maladi, S.M., Henderson, K.L.D., Coats, J.R., 2005. Phytoremediation - an overview. *Critical Reviews in Plant Sciences*, 24(2): 109-122.

Arunakumara, K.K.I.U., Walpola, B.C., Yoon, M.H., 2015. Bioaugmentation-assisted phytoextraction of Co, Pb and Zn: an assessment with a phosphate- solubilizing bacterium isolated from metal-contaminated mines of Boryeong Area in South Korea. *Biotechnology Agonomy Society Environment*, 19(2):143-152.

Atagana, H.I., 2011. Bioremediation of Co-contamination of crude oil and heavy metals in soil by phytoremediation using *Chromolaena odorata* (L) King & H.E. Robinson. *Water Air and Soil Pollution*, 215(1): 261-271.

Azcón, R., Medine, A., Raldán, A., Biró, B., Vivas, A., 2009. Significance of treated agrowaste residue and autochthonous inoculates (*Arbuscular mycorrhizal* fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediation soils contaminated with heavy metals. *Chemosphere*, 75(3): 327-334.

Azcón, R., Perálvarez, M. del C., Roldán, A., Barea, J-M., 2010. Arbuscular Mycorrhizal Fungi, *Bacillus cereus*, and *Candida parapsilosis* from a Multicontaminated soil Alleviate Metal Toxicity in plant. *Microbial Ecology*, 59:668-677.

Babu, T.S., Marder, J.B., Tripuranthakam, S., Dixon, D.G., Greenberg, B.M., 2001. Synergistic effects of a photooxidized polycyclic aromatic hydrocarbon and copper on photosynthesis and plant growth: evidence that in vivo formation of reactive oxygen

species is a mechanism of copper toxicity. *Environmental Toxicology and Chemistry*, 20(6): 1351-1358.

Baker, A.J.M., 1981. Accumulators and excluders-strategies in the response of plants to heavy metals. *Journal of Plant Nutrition*, 3(1-5):643-654.

Baldrian, P., Wiesche, C.D., Gabriel, J., 2000. Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic. *Applied Environmental Microbiology*, 66:2471-2478.

Balslev, H., 1998. *Juncaceae In: The Families and genera of vascular plants*. Kubitzki K. (ed). Vol. IV. Springer, Berlin - Heidelberg - NewYork, 252-266 pp.

Barbafieri, M., Japenga, J., Romkens, P., Petruzzelli, G., Pedron, F., 2013. Protocols for Applying Phytotechnologies in Metal-Contaminated Soils. *In: Plant-Based Remediation Processes*. Gupta, D.K. (Ed.). Springer, Verlag, Berlin, Heidelberg, 19-37pp.

Barbier, E.B. Hacker, S.D., Kennedy, C., Koch, E.W. Stier, A.C., Silliman, B.R. 2011. The value of estuarine and coastal ecosystem services. *Ecological Monographs* 81(2): 169-193.

Batty, L.C. and Anslow, M., 2008. Effect of a polycyclic aromatic hydrocarbon on the phytoremediation of zinc by two plant species (*Brassica juncea* and *Festuca arundinacea*), *International Journal of Phytoremediation*. 10(3):236-251.

Braud, A., Jézéquel, K., Vieille, E., Tritter, A., Lebeau, T., 2006. Changes in extractability of Cr and Pb in a polycontaminated soil after bioaugmentation with microbial producers of biosurfactants, organic acids and siderophores. *Water Air & Soil Pollution: Focus* 6(3-4): 261-279.

Braud, A., Jézéquel K., Bazor, S., Lebeau, T., 2009. Enhanced phytoextraction of an agricultural Cr- and Pb- Contaminated soil by bioaugmentation with siderophore-producing bacteria. *Chemosphere* 74(2): 280-286.

Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal Microbiology*, 46(3): 237-245.

Burken, J.G., 2003. Uptake and metabolism of organic compounds: Green liver model. *In: Phytoremediation: transformation and control of contaminants*. McCutcheon, S.C., Schnoor, J.L. (Eds.). John Wiley & Sons Inc., Hoboken, New Jersey, 59-84pp.

Caçador, I., Vale, C., Catarino, F., 2000. Seasonal Variation of Zn Pb, Cu and Cd concentrations in the root- sediment system of *Spartina* and *Halimione portucacoides* from Tagus estuary salt marshes. *Marine Environmental Research*, 49:279-290.

Caçador, I. and **Duarte, B.**, 2011. Salt marshes: An interesting ecosystem to study phytoremediation. *In: Handbook of phytoremediation*. Golubev, I.A. (ed.), Nova Science publishers, New York, 723-736 pp.

Çakmakçı, R., Donmez, F., Aydin, A., Sahin, F., 2006. Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. *Soil Biology Biochemistry*, 38(6): 1482-1487.

Castro, R., Pereira, S., Lima, A., Corticeiro S., Válega, M., Preira, E., Duarte,, Figueira, E., 2009. Accumulation, distribution and cellular partitioning of mercury in several halophytes of a contaminated salt marsh. *Chemosphere*, 6(10):1348-1355.

Chapman, P.M., 2007. Determining when contamination is pollution – Weight of evidence determinations for sediments and effluents. *Environment International* 33(4): 492-501.

Chen, Y.X., Lin, Q., He, Y.F., Tian, G.M., 2004. Behavior of Cu and Zn under combined pollution of 2,4-dichlorophenol in the planted soil. *Plant and Soil*, 261(1-2): 127-134.

Chen, J., Xu Q.X, Su,Y., Shi Z.Q., Han, F.X., 2013. Phytoremediation of organic polluted soil. *Journal Bioremediation & Biodegradation*, 4(3): 1000-1132.

Cheng, S., Grosse, W., Karrenbrock, F., Thennessen, M., 2002. Efficiency of constructed wetlands in decontamination of water polluted by heavy metals. *Ecological Engineering*, 18:317-325.

Chigbo, C., Batty, L., Bartlett, R., 2013. Interactions of copper and pyrene on phytoremediation potential of *Brassica juncea* in copper-pyrene co-contaminated soil. *Chemosphere*, 90(10):2542-2548.

Chigbo, C., and **Batty** L., 2015. Chelate-Assisted Phytoremediation of Copper-pyrene-Contaminated Soil Using *Z.mays*. *Water Air Soil Pollut*, 226(3):74-84.

Conesa, H.M., Evangelou, M.W.H., Robinson, B.H., Schulin, R., 2012. A critical view of current state of phytotechnologies to remediate soils: still a promising tool? *The Scientific World Journal*. doi:10.1100/2012/173829.

Costa, J.C., Arsénio, P., Monteiro-Henriques, T., Neto, C., Pereira, E., Izco, J., 2009. Finding the boundary between Eurosiberian and Mediterranean salt marshes. *Journal of Coastal Research*, Special Issue, 56(2): 1340-1344.

Couto, M.N.P.F., Borges, J.R., Guedes, P.R., Almeida, R., Monteiro, E., Almeida, C.M.R., et al., 2012. An improved method for determination of petroleum hydrocarbons from soil using a simple ultrasonic extraction and Fourier transform infrared spectrophotometry. *Petroleum Science Technology* <http://dx.doi.org/10.1080/10916466.2011.587383>.

Das, N. and **Chandran**, P., 2011. Microbial Degradation of Petroleum Hydrocarbon Contaminants:An Overview. *Biotechnology Research International*, 2011:1-13.

Davis , L.C., Castro-Diaz, S., Zhang, Q., Erickson, L.E., 2002. Benefits of vegetation for soils with organic contaminants. *Critical reviews in plant sciences* 21:457-491.

De Lange, H.J., Paulissen, M.P.C.P., Slim, P.A., 2013. “Halophyte filters”: the potential of constructed wetlands for application in saline aquaculture. *International Journal of Phytoremediation*, 15(4):352–364.

De Souza, M.P., Huang, C.P.A., Chee, N., Terry, N.,1999. Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. *Planta*, 209(2):259– 263.

Dietz, K-J., Baier, M., Kramer, U., 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: *Heavy metal stress in plant: from molecules to ecosystem*. Prasad, M.N.V., Hagemeyer, J. (eds.) , Springer-Verlag, Berlin, 73-97pp.

Dobbler, R., Saner, M., Bachofen, R., 2000. Population changes of soil microbial communities induced by hydrocarbon and metal contamination. *Bioremediation Journal* 4(1): 41–56.

Doelman, P., Jansen, E., Michels, M., Van, Til, M., 1994. Effect of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biology fertility of soils*, 17:177-184.

Dordio, A.V., and **Carvalho**, A.J.P., 2013. Organic xenobiotics removal in constructed

wetlands, with emphasis on the importance of the support matrix. *Journal of Hazardous Materials*, 252-253:272-292.

Duffus, J.H., 2002. Heavy metals - A meaningless term? *Pure and Applied Chemistry*, 74(5):793-807.

El-Deeb, B., and **Altalhi**, A.D., 2009. Degradative plasmid and Heavy metal Resistance Plasmid Naturally Coexist in Phenol and Cyanide Assimilating Bacteria. *American journal Biochemistry and Biotechnology*, 5(2):84-93.

Figueira, E., Freitas R., Pereira, E., Duarte, A., 2012. Mercury uptake and allocation in *Juncus maritimus*. Implications phytoremediation and restoration of a mercury contaminated salt marsh. *Journal environmental Monitoring*, 14(8): 2181-2188.

Gaur, N., Flora, G., Yadav, M., Tiwari, A., 2014. A review with recent advancements on bioremediation- based abolition of heavy metals. *Environmental Science: Processes & impacts*, 16(2): 180-193.

Gentry, T.J., Rensing, C., Pepper, I.L., 2004. New approaches for bioaugmentation as a remediation technology. *Environmental Science and Technology*, 34(5): 447-494.

Gerhardt, K.E., Huang, X.D., Glick, B.R., Greenberg, B.M., 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science*, 176(1): 20-30.

Glick, B.R., 2010. Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances*, 28(3):367-374.

Guala, S.D., Vega, F.A., Covelo, E.F., 2010. The dynamics of heavy metals in plant-soil interactions. *Ecological Modelling*, 221(8): 1148-1152.

Gutiérrez-Ginés, M.J., Hernández, A.J., Pérez-Leblic, M.I., Vangronsveld, J., 2014. Phytoremediation of soils co-contaminated by organic compounds and heavy metals: Biossays with *Lupinus luteus* L. and associated endophytic bacteria, *Journal of Environmental Management*, 143:197-207.

Guillon-Philippe, A., Masotti, V., Claeys-Bruno, M., Malleret, L., Coulomb, B., Prudent, P., Höhener, P., Petit, M-E., Sergent, M., Iaffont-Schwob, I., 2015. Impact of organic pollutants on metal and As uptake by halophyte species and consequences for constructed wetlands design and management, *Water Research*, 68:328-341.

Guo, J. H., Qi, H.Y., Guo, Y.H., Ge, H.L., Gong L.Y., Zhang, L.X., 2004. Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biological control*, 29(1): 66-72.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, 53(366):1-11.

Hechmi, N., Aissa, N., B., Abdenaceur, H., Jedidi, N., 2015. Uptake and Bioaccumulation of Pentachlorophenol by Emergent Wetland Plant *Phragmites australis* (Common reed) in Cadmium Co-contaminated soil. *International Journal of phytoremediation*, 17(2): 109-116.

Hoagland, D.R. and **Arnon**, D.I., 1950. The Water-Culture Method for Growing Plants Without Soil. CAES Circular 347. California Agricultural Experiment Station, California, p. 32.

Hoffman, D.R., Okon, J.L., Sandrin, T.R., 2005. Medium composition affects the degree and pattern of cadmium inhibition of naphthalene biodegradation. *Chemosphere*, 59(7):919-927.

Huesemann, M.H., 2004. Biodegradation and bioremediation of petroleum pollutants in soil. *In: Applied Bioremediation and phytoremediation*. Singh, A., Ward, O.P., (eds.), Springer, Verlag-Berlin-Heidelberg-New York, 13-29 pp.

ITRC (Interstate Technology & Regulatory Council). 2009. Phytotechnology Technical and Regulatory Guidance and Decision Trees, Revised. PHYTO-3. Washington, D.C.: Interstate Technology & Regulatory Council, Phytotechnologies Team, Tech Reg Update. www.itrcweb.org ; Accessed 13 April 2015.

Jain, P.K., Gupta,V.K., Gaur,R.K., Lowry, M., Jaroli, D.P., Chauhan, U.K., 2011. Bioremediation of Petroleum oil Contaminated Soil and Water. *Research Journal of Environmental Toxicology* 5(1):1-26.

Jetiyanon, K., and **Kloepper**, J.W., 2002. Mixture of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological control*, 24: 285 -291.

Jurtshuk, P. Jr., 1996. Bacterial Metabolism. *In: Medical Microbiology*, 4th ed., Baron, S. (ed), Galveston (TX): University of Texas Medical Branch at Galveston, Chapter 4.

Kabata-Pendias, A., 2011. Trace Elements in Soils and Plants, 4th ed. Taylor and Francis Group, Boca Raton, 505p.

Kachur, A.V., Koch, C.J., Biaglow, J.E., 1998. Mechanism of copper catalyzed oxidation of glutathione. *Free Radical Research*, 28(3):259-269.

Kang, J.W., 2014. Removing environmental organic pollutants with bioremediation and phytoremediation. *Biotechnology letters*, 36(6): 1129-1139.

Karthikeyan, R., and **Kulakow**, P.A., 2003. Soil Plant Microbe Interactions in Phytoremediation. *In: Phytoremediation: Advances in Biochemical Engineering/biotechnology*. Scheper,T. (ed.). Vol .78. Springer-Verlag, Berlin, Heidelberg, 53-64 pp.

Khan, S., Hesham, A.El-Latif, Qing, G., Shuang, L He, J., 2009. Bioremediation of pyrene and catabolic genes in contaminated soils cultivated with *Lolium multiflorum* L.. *Journal of Soils and Sediments*, 9(5): 482-491.

Kirby, M.F. and **Law**, R. J., 2010. Accidental spills at sea – Risk, impact, mitigation and the need for co-ordinated post – incident monitoring. *Marine Pollution Bulletin* 60(6):797-803.

Kirpichnikova, TA, Manceau, A., Spadini, L., Panfili, F., Marcus, M.A., Jacquet,T., 2006. Speciation and solubility of heavy metals in contaminated soil using X-ray microfluorescence, EXAFS spectroscopy, chemical extraction, and thermodynamic modeling. *Geochimica et Cosmochimica Acta*, 70(9-70):2163-2190.

Lampis, S., Ferrari, A., Cunha-Queda, A.C.F., Alvarenga, P., Gregorio, S.D., Vallini, G., 2009. Selenite resistant rhizobacteria stimulate SeO_3^{2-} phytoextraction by *Brassica juncea* in bioaugmentation water-filtering artificial beds. *Environmental Science and Pollution Research*, 16(6): 663-670.

Lampis, S., Santi, C., Ciurli, A., Andreolli, M., Vallini, G., 2015. Promotion of arsenic phytoextraction efficiency in the fern *Pteris vittata* by the inoculation of As-resistant

bacteria: a soil bioremediation perspective. *Frontiers in plant science*, 6:1-12.
<http://dx.doi.org/10.3389/fpls.2015.00080>

Lasat, M. M., 2000. Phytoextraction of metals from contaminated soil: a review of plant/soil/metal interaction and assessment of pertinent agronomic issues. *Journal of Hazardous Substance Research*, 2(5): 1-25.

Lefeuvre, J.C., Laffaille, P., Feunteun, E., Bouchard, V., Radureau, A., 2003. Biodiversity in salt marshes: from patrimonial value to ecosystem functioning. The case study of the Mont-Saint-Michel bay. *Comptes Rendus Biologies*, 326 (Suppl. 1): 125-131.

Leigh, M.B., Fletcher, J.S., Fu, X., Schmitz, F.J., 2002. Root turnover: an important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants, *Environ Sci Technol*, 36(7): 1579-1583.

Li, W.C., and **Wong, M.H.**, 2012. Interaction of Cd/Zn hyperaccumulating plant (*Sedum alfredii*) and rhizosphere bacteria on uptake and removal of phenanthrene. *Journal of Hazardous Materials*, 209-210:421-433.

Lin, Q Wang, Z., Ma, S., Chen, Y., 2006. Evaluation of dissipation mechanisms by *Lolium perenne* L., and *Raphanus sativus* for pentachlorophenol (PCP) in copper co-contaminated soil. *The Science of the Total Environment*. 368(2-3): 814-822.

Lin, Q., Shen, K.L., Zhao, H.M., Li, W.H., 2008. Growth response of *Zea mays* L. in pyrene-copper co-contaminated soil and the fate of the pollutants. *Journal Hazardous Materials*, 150(3): 515-521.

Lin, Q., and **Mendelssohn, I.A.**, 2009. Potential of restoration and phytoremediation with *Juncus roemerianus* for diesel-contaminated coastal wetlands. *Ecological Engineering*, 35:85-91.

Liu, J.G., Li, G.H., Shao W.C., Xu, J.K., Wang, D.K., 2010. Variations in uptake and translocation of copper, chromium and nickel among nineteen wetland plant species. *Pedosphere*, 20(1): 96-103.

Lodewyckx, C., Taghavi, S., Mergeay, M., Vangronsveld, J., Clijsters, H., van der Lelie, D., 2001. The effect of recombinant heavy metal-resistant endophytic on heavy metal uptake by their host plant. *International Journal of Phytoremediation*, 3(2):173-187.

Lone, M.I., He, Z., Stoffella, P.J., Yang, X., 2008. Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. *Journal of Zhejiang University – SCIENCE B*, 9(3): 210-220.

Long, E. R., MacDonald, D. D., Smith, S. L., Calder, F. D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environmental Management* 19(1): 81-97.

Lorah, M.M., Majcher, E.H., Jones, E.J., Voytek, M.A., 2008. Report. *In*: U.S.G.S.S.I (Ed.), Microbial Consortia Development and Microcosm and Column Experiments for Enhanced Bioremediation of Chlorinated Volatile Organic Compounds, West Branch Canal Creek Wetland Area, Aberdeen Proving Ground, Maryland. U.S. Geological Survey, Reston, Virginia, p. 79.

Ma, Y., Prasad, M.N.V., Rajkumar, M., Freitas, H., 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils *Biotechnology Advances*, 29(2):248-258.

Macaulay, B.M. and **Rees, D.**, 2014. Bioremediation of oil spills: a review of challenges for

research advancement. *Annals of Environmental Science*, 8:9-37.

Machado, A., Magalhães, C., Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., 2012. Microbial communities within saltmarsh sediments: composition abundance and pollution constraints. *Estuarine, Coastal and Shelf Science*, 99: 145-152.

Manivasga, L., Vijayorengam, P., Balamurugan, S., Thiyagarajan, G., 2011. Effect of copper on growth, dry matter yield and nutrient content of *Vigna Radata* (L.) Wilczek. *The Journal of Physiology*, 3: 53-63.

Manousaki, E. and **Kalogerakis**, N., 2011. Halophytes–An Emerging Trend in Phytoremediation. *International Journal of Phytoremediation*, 13(10):959-969.

Marmiroli, N., Marmiroli, M., Maestri, E., 2006. Phytoremediation and phytotechnologies: a review for the present and the future. In: *Soil and Water Pollution Monitoring, Protection and Remediation*. Twardowska, I., Allen, H.E., Häggblom, M.M., (Eds.). NATO - Science Series, 69:403–416.

Mattina, M. J. I., Lannucci-berger, W., Musante, G., White, J. C. 2003. Concurrent plant uptake of heavy metals and persistent organic pollutants from soil. *Environmental Pollution*, 124(3): 375–378.

Meagher, R.B., 2000. Phytoremediation of toxic elemental and organic pollutants. *Plant Biology*, 3(2):153-162.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Nadia, R., 2011. Bioremediation approaches for organic pollutants: A critical perspective. *Environment International*, 37(8):1362-1357.

Mendez, O. M., and Maier, R.M., 2008. Phytostabilization of mine tailings in arid and semiarid environments - an emerging remediation technology. *Environmental Health Perspectives* 116(3): 278–283.

Moreira, I.T.A., Oliveira, O.M.C., Triguís, J.A., Queiroz, A.F.S., Barbosa, R.M., Anjos, J.A.S.A., Reyes, C.Y., Silva, C.S., Trindade, M.C.L.F., Rios, M.C., 2013. Evaluation of the effects of metals on biodegradation of total petroleum hydrocarbons. *Microchemical Journal*, 110: 215-220.

Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., Vasconcelos M.T.S.D., 2005. Exudation of organic acids by a marsh plant and implications on trace metal availability in the rhizosphere of estuarine sediments. *Estuarine Coastal and Shelf Science*, 65:191-198.

Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., Vasconcelos, M.T.S.D., 2008. Salt marsh plants (*Juncus maritimus* and *Scirpus maritimus*) as sources of strong complexing ligands. *Estuarine Coastal and Shelf Science* 77(1): 104-112.

Mucha, A.P., Almeida, C.M.R., Magalhães, C.M., Vasconcelos M.T.S.D., Bordalo, A.A., 2011. Salt marsh plant-microorganism interaction in the presence of mixed contamination. *International Biodeterioration and biodegradation* 65(2):326-333.

Mucha, A.P., Teixeira, C., Reis I., Magalhães, C., Bordalo, A.A., 2013. Response of a salt marsh microbial community to metal contamination. *Estuarine Coastal and Shelf Science*, 130: 81-88.

Nie, M., Wang, Y., Yu, J., Xiao, M., Jiang,L., Yang, J., Fang, C., Chen, J., Li, B., 2011. Understanding Plant-Microbe Interactions for Phytoremediation of Petroleum - Polluted Soil. *PLoS ONE* 6(3): e17961.

Nies, D.H., 1999. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology*, 51(6):730-750.

Nnadi, E.O. and Chigbo, C., 2014. Exploring potential of using phytoremediation for co-contaminated soils. *In: Heavy metal remediation: transport and accumulation in plants.* Gupta, D.K., Chatterjee, S. (eds.). Nova Science Publishers. Inc., 201-218pp.

Nunes da Silva, M.A., 2012. Contribution of the plant / rhizosediment system to the phytoremediation of metals. *Disertação de Mestrado em Toxicologia e Contaminação Ambientais.* Instituto de Ciências Biomédicas Abel Salazar. Universidade do Porto, Porto. 99pp.

Nunes da Silva, M., Mucha, A.P., Rocha A.C., Teixeira, C., 2014. A strategy to potentiate Cd phytoremediation by saltmarsh plants – Autochthonous bioaugmentation. *Journal of Environmental Management*, 134:136-144.

Nwoko, C.O., 2010. Trends in phytoremediation of toxic elemental and organic pollutant. *African Journal of Biotechnology*, 9(37):6010-6016.

Olaniran, A.O., Balgobind, A., Pillay, B., 2011. Quantitative assessment of the toxic effect of heavy metals on 1,2 – dichloroethane biodegradation in co-contaminated soil under aerobic condition. *Chemosphere*, 85(5):839-847.

Olaniran, A.O., Balgobind, A., Pillay, B., 2013. Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. *International Journal of Molecular Sciences*, 14(5):10197-10228.

Oliveira, T., Mucha, A.P., Reis I., Rodrigues, P., Gomes, C.R., Almeida, C.M.R., 2014. Copper phytoremediation by salt marsh plant (*Phragmites australis*) enhanced by autochthonous bioaugmentation. *Marine Pollution Bulletin*, 88(1-2):231-238.

Oliveira, V., Gomes, N.C.M., Almeida, A., Silva, A.M.S., Silva, H., Cunha, A., 2015. Microbe-assisted phytoremediation of hydrocarbons in estuarine environments *Microbial Ecology* 69(1):1-12.

Panagos, P., van Liedekerke, M., Yigini, Y., Montanarella, L., 2013. Contaminated sites in Europe: review of the current situation based on data collected through a European network. *Journal of Environmental and Public*, 11 pages.

Peixoto, R.S., Vermelho, A.B., Rosado, A.S., 2011. Petroleum-Degrading Enzymes: Bioremediation and New Prospects. *Enzymes Research* 2011:1-7.

Peng, K., Luo, C., Lou, L., Li, X., Shen, Z., 2008. Bioaccumulation of heavy metals by the aquatic plants *Potamogeton pectinatus* L. and *Potamogeton malaianus* Miq. and their potential use for contamination indicators and in wastewater treatment. *The science of the Total Environment*, 392(1):22-29.

Pepper, I.L., Gentry, T.J., Newby, D.T., Roane, T.M., Josephson, K.L., 2002. The role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soils. *Environmental Health Perspectives*, 110(6): 943-946.

Phillips, L.A., Greer, C.W., Farrell, R.E., Germida, J.J., 2012. Plant root exudates impact the hydrocarbon degradation potential of a weathered –hydrocarbon contaminated soil. *Applied Soil Ecology*, 52(1): 56-64.

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

Prapagdee, B., Chanprasert, M., Mongkolsuk, S., 2013. Bioaugmentation with cadmium-resistant plant growth-promoting rhizobacteria to assist cadmium phytoextraction by *Helianthus annuus*. *Chemosphere*, 92(6): 659-666.

Rajkumar, M., Ae, N., Prasad, M.N.V., Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in Biotechnology*, 28(3):142-149.

Rajkumar, M., Sandhya, S., Prasad, M.N.V., Freitas, H., 2012. Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnology Advances*, 30(6): 1562-1574.

Rani, R., and Juwarkar, A., 2013. Interactions between plant growth promoting microbes and plants: Implications for microbe-assisted phytoremediation of metal-contaminated soil. In: *Recent Advances Towards Improved Phytoremediation of Heavy Metal Pollution*. Leung, D.W.M. (Ed.), pp. 3-39. Available in database: eBook Academic Collection (EBSCOhost)

Rauret, G.; López-Sánchez, J. F.; Sahuquillo, A.; Rubio, R.; Davidson, C.; Ure, A.; Quevauviller, Ph., 1999. Improvement of the BCR three step sequential extraction procedure prior to the certification of the new sediment and soil reference materials. *Journal of Environmental Monitoring*, 1(1):57-61.

Reborela, R., and **Caçador**, I., 2007. Halophyte vegetation influences in salt marsh retention capacity for heavy metals. *Environmental Pollution*, 146:147-154.

Reichenauer T.G., and **Germida**, J.J., 2008. Phytoremediation of organic contaminants in soil and groundwater. *Sustainable Chemistry & Green Chemistry*, 1(8-9):708-717.

Rengel, Z., Zhang, ZH., Meney, K., Pantelic, L., Tamanovic, R., 2011. Polynuclear aromatic hydrocarbons (PAHs) mediate cadmium toxicity to an emergent wetland species. *Journal of Hazardous Materials*, 189(1-2): 119-126.

Ribeiro, H., Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., 2011. Hydrocarbon degradation potential of salt marsh plant-microorganisms associations. *Biodegradation*, 22(4):729-739.

Ribeiro, H.M.S.R., 2013. Petroleum hydrocarbons bioremediation in a temperate salt marsh: plant-microorganisms interactions. Ph.D. Thesis. Instituto de Ciências Biomédicas Abel Salazar, University of Porto. Porto.187pp.

Ribeiro, H., Almeida, C.M.R., Mucha, A.P., Bordalo, A.A., 2013a. Influence of different salt marsh plants on hydrocarbon degrading microorganisms abundance throughout a phenological cycle. *International Journal of Phytoremediation*, 15(8):715-728.

Ribeiro, H., Almeida, C.M.R., Mucha, A.P., Teixeira, C., Bordalo, A.A., 2013b. Influence of natural rhizosediments characteristics on hydrocarbons degradation potential of microorganisms associated to *Juncus maritimus* roots. *International Biodeterioration & Biodegradation*, 84:86-96.

Ribeiro, H., Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., 2014. Potential of phytoremediation for the removal of petroleum hydrocarbons in contaminated salt marsh sediments. *Journal of environmental Management*, 137:10-15.

Riis, V., Babel, W., Pucci, O.H., 2002. Influence of heavy metals on the microbial degradation of diesel fuel. *Chemosphere*, 49(6): 559-568.

Roane, T.M., Josephson, K.L., Pepper, I.L., 2001. Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. *Applied and Environmental Microbiology*,

67(7): 3208-3215.

Ryan, B.M., Kirby, J.K., Degryse, F., Harris, H., McLaughlin, M.J., Scheiderich, K., 2013. Copper speciation and isotopic fractionation in plant: uptake and translocation mechanisms. *New Phytologist*, 199(2):367-378.

Said, W.A., and **Lewis**, D.L., 1991. Quantitative Assessment of the Effects of Metals on Microbial Degradation of Organic Chemicals. *Applied and Environmental Microbiology*, 57(5):1498-1503.

Salt, D.E, Smith, R.D., Raskin, I., 1998. Phytoremediation, *Annual Review of Plant Physiology Plant Molecular Biology*, 49: 643-668.

Sandrin, T.R., Chech, A.M., Maier, R.M., 2000. A Rhamnolipid Biosurfactant Reduces Cadmium Toxicity during Naphthalene Biodegradation. *Applied and Environmental Microbiology*, 66(10): 4585-4588.

Sandrin, T.R., and **Maier**, R.M., 2003. Impact of metals on the biodegradation of organic pollutants. *Environmental Health Perspectives*, 111(8):1093-1101.

Sandrin, T.R. and **Hoffman**, D.R., 2007. Bioremediation of organic and metal co-contaminated environments: effects of metal toxicity, speciation and bioavailability on biodegradation. In: *Environmental Bioremediation Technologies*. Singh, S.N., Tripathi, R.D. (Eds.) Springer- Verlag, Berlin, Heidelberg, pp 1-34.

Sarkar, D., Ferguson, M., Datta, R., Birnbaum, S., 2005. Bioremediation of petroleum hydrocarbons in contaminated soil: comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. *Environmental pollution*, 136:187-195.

Shank, G.C., Skrabal, S.A., Whitehead, R.F., Kieber, R.J., 2004. Fluxes of strong Cu-complexing ligand from sediments of an organic-rich estuary. *Marine Chemistry*, 60(2): 349-358.

Sheoran, V., Sheoran, A.S., Poonia, P., 2011. Role of hyperaccumulators in phytoextraction of metals from contaminated mining sites: a review. *Environmental Science and Technology*, 41(2): 168-214.

Shilev, S., Kuzmanova, I., Sancho, E., 2009. Phytotechnologies: how plants and bacteria work together. In: *Uncertainties in Environmental Modelling and Consequences for Policy Making*. Baveye, P., Laba, M., Mysiak, J. (Eds.), Springer- Netherlands, pp 385-397.

Singer, A.C., Bell, T., Heywood, C.A., Smith, J.A.C., Thompson, I.P., 2007. Phytoremediation of mixed-contaminated soil using the hyperaccumulator plant *Alyssum lesbiacum*: Evidence of histidine as a measure of phytoextractable nickel. *Environmental Pollution*, 147(1): 74-82.

Singh, A., Parmar, N., Kuhad, R.C., Ward, O., P., 2011. Bioaugmentation, Biostimulation and Biocontrol in soil biology. In: *Bioaugmentation, biostimulation and Biocontrol*. Singh A. and Parmar, N. (eds.), Vol 28. Springer, Verlag, Berlin, Heidelberg, 1-26 pp.

Skrabal, S.A., Donat, J.R., Burdige, D.J., 1997. Fluxes of copper-complexing ligands from estuarine sediments. *Limnology and Oceanography*, 42(5):992-996.

Skrabal, S.A., Donat, J.R., Burdige, D.J., 2000. Pore water distributions of dissolved copper and copper-complexing ligands in estuarine and coastal marine sediments. *Geochimica et Cosmochimica Acta* 64(11): 1843-1857.

Stroud, J.L., Paton, G.I., Semple, K.T., 2007. Microbe-aliphatic hydrocarbon interactions in

soil: implications for biodegradation and bioremediation. *Journal of Applied Microbiology* 102(5):1239-1253.

Sun, Y.B., Zhou, Q.X., Xu, Y.M., Wang, L., Liang, X.F., 2011. Phytoremediation for co-contaminated soils of benzo[a]pyrene (B[a]P) and heavy metals using ornamental plant *Tagetes patula*. *Journal of Hazardous Materials*, 186(2-3):2075-2082.

Sun, L., Liao, X., Yan, X., Zhu, G., Ma D., 2014. Evaluation of heavy metal and polycyclic aromatic hydrocarbons accumulation in plants from typical industrial sites: potential candidate in phytoremediation for co-contamination. *Environmental Science and Pollution Research*, 21(21):12494-12504.

Sundby, B., Caetano, M., Vale, C., Gobell, C., Luther, G.W., III, Nuzzio, D., 2005. Root-induced cycling of lead in salt marsh sediments. *Environmental Science & Technology*, 39(7): 2080-2086.

Tak, H.I., Ahmad, F., Babalola, O.O., 2013. Advances in the application of plant growth-promoting rhizobacteria in the phytoremediation of heavy metals. *In: Reviews of environmental contamination and toxicology*. Whitacre, D.M. (ed.). Vol 223. Springer Science Business Media, New York, 33-47 pp.

Tangahu, B.V., Abdullah, S.R., Basri, H., Idris, M., Anuar, N., Mukhlisin, M., 2011. A review on heavy metals (As, Pb, and Hg) Uptake by plants through phytoremediation. *International Journal of Chemical Engineering*. Article ID 939161, 31 pages.

Teixeira, C., Almeida, C.M.R., Nunes da Silva, M., Bordalo, A.A., Mucha, A.P., 2014. Development of autochthonous microbial consortia for enhanced phytoremediation of salt-marsh sediments contaminated with cadmium. *Science of the Total Environment*, 493: 757-765.

Tyagi, M., Fonseca, M.M.R., Carvalho, C.C.C.R., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation* 22:213-241.

UNEP (2003) Phytotechnologies: a technical approach in environmental management. IETC Freshwater Management Series 7. <http://www.unep.or.jp/ietc/Publications/Freshwater/FMS7/index.asp>. Accessed 19 April 2015.

Van Assche, F., and Clijsters, H., 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment* 13(3):195-206.

Van Beilen, J.B. and Funhoff, E.G., 2007. "Alkane hydroxylases involved in microbial alkane degradation". *Applied Microbial and Biotechnology*, 74(1):13-21.

Van Hamme, J.D., Singh, A., Ward, O.P., 2003. Recent advances in petroleum microbiology. *Microbiology Molecular Reviews*, 67(4):503-549.

Van Liedekerke, M., Prokop, G., Rabl-Berger, S., Kibblewhite, M., Louwagie, G., 2014. Progress in the management of contaminated sites in Europe, JRC Reference Reports European Commission, Joint Research Centre, Luxembourg, accessed 15 June 2015.

Vernberg, F.J., 1993. Salt-marsh processes: a review. *Environmental Toxicology and Chemistry* 12(12): 2167-2195.

Wang, J., Zhang, Z., Su, Y., He, F., Song, H., 2008. Phytoremediation of petroleum polluted soil. *Petroleum Science*, 5: 167-171.

- Wang, K., Zhu, Z., Huang, H., Li, T., He, Z., Yang, X., Alva, A., 2012.** Interactive effects of Cd and PAHs on contaminants removal from co-contaminated soil planted with hyperaccumulator plant *Sedum alfredii*. *Journal of Soils and Sediments* 12(4):554-564.
- Wang, W., Zhang, X., Huang, J., Yan, C., Zhang, Q., Lu, Haoliang, Liu, J., 2014.** Interactive effects of cadmium and pyrene on contaminant removal from co-contaminated sediment planted with mangrove *Kandelia obovata*. *Marine Pollution Bulletin*, 84(1-2): 306-313.
- Weis, P., Windham, L., Burke, D.J., Weis, J.S., 2002.** Release into the environment of metals by two vascular salt marsh plants. *Marine Environmental Research* 54(3): 325-329.
- Wenzel, W.W., 2009.** Rhizosphere processes and management in plant - assisted bioremediation(phytoremediation) of soils. *Plant and soil*, 321(1-2): 385-408.
- Wetzel, A., Alexander, T., Brandt, S., Haas, R., Werner, D., 1994.** Reduction by fluoranthene of copper and lead accumulation in *Triticum aestivum* L, *Bulletin of Environmental Contamination and Toxicology*, 53:856-862.
- Weyens, N., Croes, S., Dupae, J., Newman, L., van der Lelie, D., Carleer, R., Vangronsveld, J., 2010.** Endophytic improve phytoremediation of Ni and TCE co-contaminantion. *Enviromental Pollution*, 158(7): 2422-2427.
- Weyens, N., Truyens, S., Saenen, E., Boulet, J., Dupae, J., Taghavi, S., van der Lelie, D., Carlee, R., Vangronsveld, J., 2011.** Endophytes and their potential to deal with co-contamination of organic contaminants (Toluene and toxic metals (Nickel) during phytoremediation. *Journal of Phytoremediation*, 13(3): 244-255.
- White, P. J., 2012.** Heavy metal toxicity in plants. In: *Plant Stress Physiology*. Shabala S. (Ed.), CABI, Wallingford, pp 210-237.
- Windham, L., Weis, J.S., Weis, P., 2003.** Uptake and distribution of metals in two dominant salt marsh macrophytes, *Spartina alterniflora* (cordgrass) and *Phragmites australis* (common reed). *Estuarine, Coastal and Shelf Science*, 56(1): 63-72.
- Wu, G., Kang, H., Zhang, X., Shao, H., Chu, L., Ruan, C., 2010.** A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities. *Journal of Hazardous Materials*, 174(1-3): 1-8.
- Wuana, R.A., and Okieimen, F.E., 2011.** Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies foe remediation. *International Scholarly Research Network, ISRN Ecology*, 1-20 pp. doi: 10.5402/2011/402647.
- Wuana, R.A., Okieimen, F.E., Vesuwe, R.N., 2014.** Mixed contaminant interactions in soil: Implications for bioavailability, risk assessment and remediation. *African Journal of Environmental Science and Technology*. 8(12):691-706.
- Yadav, S.K., 2010.** Heavy metals toxicity in plant: An overview on the role of glutathione and phytochelatins in heavy metals stress tolerance of plants. *South African Journal of Botany*, 76:167-179.
- Yadav, A.K., Abbassi, R., Kumar, N., Satya, S., Sreekrishnan, T.R., Mishra, B.K., 2012.** The removal of heavy metals in wetland microcosms: Effects of bed depth, plant species, and metal mobility. *Chemical Engineering Journal*, 211-212: 501-507.
- Young, L.Y., Liang, W., Shor, L., Kosson, D., Rochne, K., Taghon, G., 2002.** Bioavailability of PAHs to bacteria in estuarine sediment. *Soil and Sediment Contamination*, 11(3): 488-488.

Zawierucha, I. and Malina G., 2011. Bioremediation of contaminated soils: effects of bioaugmentation and biostimulation on Enhancing biodegradation of oil. *In: Bioaugmentation, biostimulation and Biocontrol*. Singh A. and Parmar, N. (eds.). Vol 28. Springer, Verlag, Berlin, Heidelberg, 187- 202 pp.

Zhang, H., Dang, Z., Zheng, L.C, Yi, Y., 2009. Remediation of soil co-contaminated with pyrene and cadmium by growing maize (*Zea mays* L.) International Journal Science Technology, 6(2):249-258.

Zhang, Z., Rengel, Z., Meney, K, 2010. Polynuclear aromatic hydrocarbons (PAHs) differentially influence growth of various emergent wetland species. Journal Hazardous Materials, 182(1-3): 689-695.

Zhang, Z., Rengel, Z., Meney, K., Pantelic, L., Tomanovic, R., 2011. Polynuclear aromatic hydrocarbons (PAHs) mediate cadmium toxicity to an emergent wetland species. Journal of Hazardous Materials, 189(1): 119-126.

Zhang, Z., Rengel, Z., Chang, H., Meney, K., Pantelic, L., Tomanovic, R., 2012. Phytoremediation potential of *Juncus subsecundus* in soil contaminated with cadmium and polynuclear aromatic hydrocarbons (PAHs). Geoderma, 175-176: 1-8.

