Copper phytoremediation by a salt marsh plant: microorganisms’ contribution to enhance it
Copper phytoremediation by a salt marsh plant: microorganisms’ contribution to enhance it

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.

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Abstract

Estuarine areas are target of several sources of pollution that include contaminants like metals, which can accumulate in the environment, presenting a threat.

Most of the estuaries present large salt marsh areas, which represent a very important ecosystem with several functions of major importance for the organisms that live there. Therefore, it is of major importance to treat and restore these areas.

Phytoremediation (the use of plants in association with rhizosphere microorganisms) is presented as a green, environmentally-friendly alternative to recover these areas, being of well acceptance.

Bioaugmentation can be used to potentiate phytoremediation by plants through symbiotic association with rhizosphere microorganisms. However, one should consider the effects of introducing specific strains of bacteria in the site to be recovered, once they might not adapt or they can overcome the native ones. An option can be autochthonous bioaugmentation.

The aim of this work was to evaluate the potential of the salt marsh plant Phragmites australis for phytoremediation of Cu contaminated sediment and to evaluate if this potential can be enhanced by bioaugmentation with an autochthonous microorganism consortium that is resistant to Cu. A complementary aim consisted in the characterization of the microbial communities at the end of the experiment, as well as the characterization of microbial consortium used in the autochthonous bioaugmentation in terms of bacterial abundance and community structure.

Salt marsh plants with sediment attached to their roots were collected in an estuarine area and placed in vessels while an equal number of vessels were left unplanted. Sediment from both planted and unplanted vessels was contaminated with Cu (ca. 270 µg g⁻¹) and autochthonous microbial consortia resistant to Cu (prepared in the laboratory) were added to half of the vessels. Vessels were kept in greenhouses, under estuarine tidal regime simulation.

After two months, metal concentrations on plant structures and
sediments were determined, as well as metal speciation on sediments. Plants physiological parameters (chlorophylls, carotenoids and thiolic compounds) and plant biomass weight were also determined to assess possible stress signs.

Plants accumulated significant amounts of Cu (2–10 times more when comparing to the control) in all tissues although in higher amounts (7–10 times more when comparing to the control) in belowground structures. Bioaugmentation lead to a decrease in belowground structures biomass weight, probably because it slightly increased (5-10%) sediment total bioavailable Cu. However, the addition of the microbial consortia potentiated an increase in metal translocation, with higher amounts (2 times more) of Cu in the plant stems, without significant visual toxicity signs. This increase in Cu concentration was accompanied by an increase in the production of thiolic compounds in plant leaves, indicating that the plant had mechanisms to respond to the uptake of the metal.

After the two month experiment it was observed that the presence of copper in the sediment was an important factor in shaping the microbial communities. This feature was also observed in the consortia added to the vessels where the presence of copper lead to lower bacterial richness and diversity, probably due to the favored growth of specific species resistant to the metal.

Differences were not observed in sediment microbial communities' structures among treatments without and with bioaugmentation, despite the higher amount of bioavailable copper present in the sediment when the consortia were added.

In conclusion, this work demonstrated that *P. australis* is capable of phytoremediate Cu contaminated sediments and that autochthonous bioaugmentation can increase the phytoextraction potential of this plant. Therefore, phytoremediation with autochthonous bioaugmentation can be a valuable strategy for the recovery and management of moderately impacted estuaries.
Áreas estuarinas são alvo de vários tipos de poluição, que incluem contaminantes como os metais, os quais podem acumular no ambiente representando uma ameaça. A maior parte dos estuários apresenta grandes áreas de sapal que representam um ecossistema muito importante com funções de extrema importância para os organismos que lá habitam. Como tal, é essencial o tratamento e recuperação destas áreas.

A fitorremediação (o uso de plantas em associação com os organismos da rizosfera) é proposta como uma alternativa verde e amiga do ambiente para recuperar estas áreas, sendo de boa aceitação.

O bioaumento pode ser usado para potenciar a fitorremediação por parte das plantas através de associações simbióticas com os organismos da rizosfera. Contudo, deve-se ter em consideração os efeitos de introduzir estirpes específicas de bactérias, uma vez que elas podem não se adaptar ou então sobrepor-se às nativas. Uma opção pode ser o uso de bioaumento autóctone.

O objectivo deste trabalho foi avaliar o potencial da planta de sapal *Phragmites australis* para fitorremediação de sedimentos contaminados com cobre e avaliar se este potencial pode ser incrementado através de bioaumento com um consórcio de microorganismos autóctones resistentes ao cobre. Um objectivo complementar consistiu na caracterização das comunidades microbianas no final da experiência, bem como do consórcio microbiano usado no bioaumento autóctone em termos de abundância bacteriana e estrutura das comunidades.

Plantas de sapal com sedimento agregado às raízes foram colhidas numa zona estuarina e colocadas em vasos e um número igual de vasos foram deixados sem planta, apenas sedimento. Os sedimentos dos vasos plantados e não plantados foram contaminados com cobre (ca. 270 µg g⁻¹) e o consórcio microbiano autóctone (preparado no laboratório) foi adicionado a metade dos respectivos vasos. Os vasos foram mantidos em estufas, num sistema que simulava as condições de marés num estuário.
Após dois meses, as concentrações de metal nas estruturas da planta e no sedimento foram determinadas, bem como a especiação do metal nos sedimentos. Parâmetros fisiológicos da planta (clorofílias, carotenóides e compostos tiólicos) e pesos da biomassa também foram determinados para avaliar possíveis sinais de stress.

As plantas acumularam níveis significativos de cobre (2 a 10 vezes maior em relação ao controlo) em todos os tecidos, contudo, em maiores quantidades (7 a 10 vezes maior em relação ao controlo) nas estruturas subterrâneas. O bioaumento levou a um decréscimo no peso da biomassa das estruturas subterrâneas, o que provavelmente é um efeito da maior biodisponibilidade total de cobre nos sedimentos (5-10%) provocado pelo bioaumento. No entanto, a adição do consórcio microbiano potenciou um aumento na translocação do metal, com maiores concentrações (2 vezes maiores) nos caules da planta, não havendo sinais significativos de toxicidade visíveis. Este aumento da concentração de Cu foi acompanhado por um aumento na produção de compostos tiólicos nas folhas da planta, indicando que a planta teve mecanismos de resposta à captação de metal.

Após dois meses da experiência foi observado que a presença de Cu no sedimento foi um factor importante em relação a alterar as comunidades microbianas. Este efeito também foi observado nos consórcios adicionados aos vasos, onde a presença de Cu levou a uma menor riqueza bacteriana e diversidade, provavelmente devido ao crescimento favorecido de espécies específicas resistentes ao metal.

Não foram observadas diferenças nas estruturas das comunidades microbianas do sedimento entre tratamentos com e sem bioaumento, apesar das maiores quantidades de cobre biodisponível presente no sedimento quando o consórcio foi adicionado.

Como conclusão, este estudo demonstrou que *P. australis* é capaz de fitorremediar sedimentos contaminados com cobre e que o bioaumento autóctone pode aumentar o potencial de fitoextracção desta planta. Assim, fitorremediação com bioaumento autóctone pode ser uma estratégia viável para a recuperação de estuários moderadamente contaminados.
LIST OF PUBLICATIONS

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ARISA - Automated rRNA Intergenic Spacer Analysis
CIIMAR - Interdisciplinary Centre of Marine and Environmental Research
CTAB - Cetyl trimethylammonium bromide
Cu-RAMC - Copper-resistant autochthonous microbial consortia
DAPI - 4',6-diamidino-2-phenylindole
DNA - Deoxyribonucleic acid
dNTPs - Deoxynucleotide triphosphates
EOCs - Emerging organic contaminants
ERM - Effects range-median
GSH - Reduced glutathione
Kd - Partitioning coefficient
MDS - Multidimensional scaling
NTU - Nephelometric turbidity units
OTU - Operational taxonomic units
PAHs - Polycyclic aromatic hydrocarbons
PCR - Polymerase chain reaction
PGPR - Plant growth promoting rhizobacteria
POPs - Persistent organic pollutants
ROS - Reactive oxygen species
rRNA - Ribosomal ribonucleic acid
SE - Sequential extraction
TCC - Total cell counts
CHAPTER I

Introduction

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1. Introduction

1.1. Estuaries

As defined by Potter et al. (2010), “an estuary is a partially enclosed coastal body of water that is either permanently or periodically open to the sea and which receives at least periodic discharge from a river(s), and thus, while its salinity is typically less than that of natural sea water and varies temporally and along its length, it can become hypersaline in regions when evaporative water loss is high and freshwater and tidal inputs are negligible”.

As such, these systems are influenced by the tides and the mixture of both marine and fresh waters that result in the strong gradients of various environmental variations (physical and chemical) that estuaries are characterized by, namely abrupt changes in salinity, temperature, pH, oxygen, redox potential, nutrients, turbidity and amount and composition of particles (Ramos et al., 2005, and references therein). However, the characteristic that most distinguishes estuaries from marine and fresh waters is the high variability in salinity (Elliott & Quintino, 2007).
Different characteristics lead to unique and completely different estuaries from each other, and Fig. 1 shows an example of two different estuaries.

The different conditions observed in these ecosystems contribute to their status as naturally stressed environments (Elliott & Quintino, 2007).

Due to the variety of conditions that they present, estuaries are very important to the life history and development of many species. For example, to aquatic species by providing resources to rearing and feeding of and the creation of nursery grounds, but also for the preservation of bird species, including conservation of migratory routes (Chapman & Wang, 2001; Ridgway & Shimmield, 2002). They are also important in the transfer of sediments between fluvial and marine zones (Ridgway & Shimmield, 2002).

Species distribution in estuaries is primarily influenced by salinity, but also by other factors such as anthropogenic pollution, which is an additional stress factor for them (Chapman & Wang, 2001).

Therefore, the presence of contaminants in these areas will not only affect all species present, but will also allow the transfer of these pollutants to other areas. In order to avoid such situations, it is of great importance to fully understand the contamination present, so it is possible to apply methods to remove the contaminants.

Due to their privileged location, many estuarine areas are targets of contamination from several sources, being often considered sinks of pollutants (Almeida et al., 2004). The contamination they are target can result from municipal and industrial wastes, agricultural runoff, recreational boating, shipping traffic, coastal development but also runoff after rain events and human activities upstream (Lytle & Lytle, 2001), all contributing to the degradation of these naturally stressed ecosystems.

Worldwide, there is the concern about coastal contamination from several sources including metals, petroleum and polycyclic aromatic hydrocarbons (PAHs) (Readman et al., 2002), other persistent organic pollutants (POPs) (Zhang et al., 2003) and also emerging organic contaminants (EOCs), such as antibiotics among others (Jiang et al., 2014).
When it comes to metals, for example, China is one of the coastal countries that face severe threats due to the increasingly metal pollution observed, posing a major concern not only to ecological impacts but also for human exposure (Pan & Wang, 2012).

Estuaries are dynamic, complex and unique systems, with continually changing characteristics (Chapman & Wang, 2001), which points out to the importance in studying these ecosystems over long periods and the difficult in creating general concepts that apply to all.

1.1.1. Salt marshes

A salt marsh is a buildup of sediments, either from sea or river origin, in sheltered parts of coastal areas.

Salt marshes represent the final stage in the leveling of marine delta plains or the filling of depressions, embayments and other irregularities along coasts and might be present in several coastal systems, such as barrier islands, saline to brackish estuaries or deltas and embayments (Frey & Basan, 1978).

![Fig. 2. Example of a salt marsh. Retrieved from: http://commons.wikimedia.org/wiki/File:Salcott_Creek-Old_Hall_Marshes_Salt_Marsh-_geograph.org.uk--218032.jpg.](http://commons.wikimedia.org/wiki/File:Salcott_Creek-Old_Hall_Marshes_Salt_Marsh-_geograph.org.uk--218032.jpg)
Fig. 2 illustrates a salt marsh area, with the characteristic vegetation.

In an early stage of a salt marsh development, salt tolerant plants begin to colonize the area and promote the trapping of sediments, resulting in an upward development of the salt marshes (Boorman et al., 1999). However, these ecosystems are also very influenced by several factors such as waves and tidal regimes and the input of sediment that can lead to their disappearing (Adam, 1993). Therefore, these are ecosystems in constant change and evolution due to all the variables they subjected to.

In addition to trapping sediment, salt marshes are also able to trap various types of contaminants, acting as a sink for pollutants, similarly to estuaries, as seen before (Almeida et al., 2004; Caçador et al., 2007).

However, there should be the concern that this sinks might eventually become a source of pollutants.

Salt marshes are among the most productive systems on earth (Boorman et al., 1999) and also have several functions which make them a very important ecosystem with the need to be preserved.

For example, they provide a dynamic buffer between land and sea, playing a major role by providing an effective and sustainable method for coastal defense from erosion; are a vital source of nutrients and organic matter to marine communities; salt marshes also provide spawning sites and nursery areas for many fish species as well as feeding, roosting and nesting areas for a wide range of bird species (Boorman et al., 1999).

Most of the estuaries present large areas of salt marsh that are colonized by different species of plants (Almeida et al., 2011).
As a result of the different factors that salt marsh environments are affected by, salt marsh plants are exposed to a high variability of extreme conditions that will affect their distribution across these areas (Fig. 3) and, consequently, salt marshes are characterized by low flora diversity and by the zonation of distinct plant species (Caçador et al., 2007). Of these factors, the one that has most been associated with the distribution of salt marsh plants is the variability in the plants physiological tolerances for salinity and flooding, which are both related to the tides (Hladik & Alber, 2014). However, sediment structure, soil redox potential, nutrient levels, competitive ability and anthropogenic factors also play an important role (Caçador et al., 2007; Huckle et al., 2000).

Nevertheless, these factors alone are not able to explain the spatial distribution of halophytes, thus a combination of multiple factors should be considered (Silvestri et al., 2005).
Salt marsh plants play an important role within these areas and, for the scope of this work, the most important is their shown ability to uptake and phytoremediate metals (Weis & Weis, 2004), resulting in a reduction of metals levels in sediments and, consequently, in a reduction in the potential toxic effects to the organisms present in these habitats (Almeida et al., 2009).

However, different plants have different behaviors and characteristics and so studies should be addressed in order to find out how specific salt marsh plants have the potential to be used in the phytoremediation process.

1.1.2. *Phragmites australis*

*Phragmites australis* is one of the plants that can be found in estuaries salt marshes (Fig. 4).

*Fig. 4. A – P.australis anatomy (retrieved from: http://www.pfaf.org/Admin/PlantImages/PhragmitesAustralis.jpg) and B – specimens growing in a natural area (retrieved from: http://upload.wikimedia.org/wikipedia/commons/c/cf/Phragmites_australis.jpg).*

*P. australis*, commonly known as the common reed, is a rhizomatous perennial macrophyte, characteristic of shallow water and wet soil (wetlands), having a worldwide range (URI, 2007). In Portugal, it is found in wetlands throughout the country, with exception of high altitudes.
This plant is suitable for light (sandy), medium (loamy) and heavy (clay) soils, can tolerate wide ranges of pH (from acid to very alkaline), and also endures wide ranges of salinity, including exposure to sea water (URI, 2007), pointing out to the variety of areas in which the plant is present. Although *P. australis* has been historically located in the high marsh area, near the interface with land, now it is commonly found in lower marsh areas (Windham & Lathrop, 1999).

The common reed is able to produce a large quantity of biomass and has been shown to alter soil properties, which are characteristics that favor the invasive potential that the plant has in some regions (Catling & Mitrow, 2011; Saltonstall *et al.*, 2004; Windham & Lathrop, 1999).

In addition, *P. australis* has shown the ability to resist to several contaminants that affect estuarine salt marshes. For instance, it has shown to accumulate significant amounts of metals, including copper, on its belowground structures and to concentrate metals in its rhizosediment (Almeida *et al.*, 2011).

1.2. Metals

1.2.1. Metals in the environment

The most part of all metals that are present today in the environment have been biogeochemically cycled since the formation of the Earth 4.5 billion years ago. However, there are several processes derived from anthropogenic activities that have increased the input of metals to the environment (Garrett, 2000).

Therefore, metals are naturally present in estuarine environments at trace levels, being essentials to the normal development of these environments as well as for the organisms therein (Bryan, 1971). Despite this, and due to their non-degradable nature, metals tend to accumulate in environmental
compartments for long residence times (Aly et al., 2013), presenting a threat to both human health and the environment.

Industrial and agricultural activities are potential sources of metals to estuarine and riverine areas (Arribére et al., 2003), leading to the presence of several metals in these environments (Akçay et al., 2003; Aly et al., 2013; Arribére et al., 2003).

Once in aquatic environments metals can be separated in different fractions: water-soluble species, colloids, suspended forms and sedimentary phases (Peng et al., 2009). Metals are not removed by natural processes of decomposition, so they can be enriched and stored in sediments and, in some conditions, that corresponds to 99% of metals input (Peng et al., 2009).

Most metals are essential to organisms, including to plants, however, when presents in high concentrations metals are known to cause adverse and toxic effects, being copper an example of this (Flemming & Trevors, 1989; Bryan, 1971).

Copper is a reddish-brown metal with atomic number 29 and atomic mass 63, occurs in a metallic form or in compounds as Cu(I) or Cu(II) and is almost always extracted from ores in undergrounds or open-pit mines (Merian, 1991), being naturally found in sandstones and in minerals such as malachite and chalcopyrite (Mulligan et al., 2001). Its main uses include electrical applications, chemical and pharmaceutical compounds and alloys, among many others (Merian, 1991).

Copper is produced more than any other metal and its use has increased substantially in the past decades, mainly because of its use in fertilizers and pesticide sprays, building materials, industrial emissions, among others (Mulligan et al., 2001).
1.2.2. Metals Bioavailability

As described by Luoma (1983) (in Eggleton & Thomas, 2004), a contaminant bioavailability is “the reactivity of each contaminant with the biological interface, that will depend on the presence of other chemicals that may antagonize or stimulate uptake, and on external factors such as temperature that affect the rate of biological or chemical reactions”.

Several physical and chemical characteristics of the soil influence metal speciation and its mobility, as for example, soil pH, redox potential and, organic, carbonate, clay and oxide contents. Metals mobility is dependent on its form (Mulligan et al., 2001):

- High mobility – simple and complex cations
- Medium mobility – exchangeable cations in organic and inorganic complexes
- Slight mobility - chelated cations
- Metals in organic or mineral particles are only mobile after decomposition
- Precipitated metals are mobile under dissolution conditions (e.g. change in pH)

Unlike freshwaters, where pH is the controlling factor, in estuaries salinity is the controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters, affecting contaminant bioavailability (Chapman & Wang, 2001).

For metals, the main binding phases include organic carbon for hydrophobic chemicals and sulfide, organic matter, and iron and manganese oxyhydroxyides (Chapman et al., 1998).

Several physical processes that occur in coastal areas can affect the partitioning and bioavailability of pollutants in estuarine sediments, such as river flow, tidal flushing, and other sediment resuspension events (Chapman & Wang, 2001).
Metals bioavailability in aquatic environment depends mainly on the partitioning behavior or binding strength of the contaminant to sediment (Eggleton & Thomas, 2004).

The partitioning coefficient (Kd) of a contaminant is defined as the ratio of a contaminant concentration in the sediment to that dissolved in the overlying or interstitial water (Chapman & Wang, 2001) and is influenced by hydrodynamics, biogeochemical processes and environmental conditions (redox, pH, salinity and temperature) (Eggleton & Thomas, 2004).

Copper has a median partition coefficient of 4.2 for the ratio sediment/water, demonstrating its affinity to sediments (Allison & Allison, 2005).

Copper binds strongly to organic matter and clay mineral, which decreases its mobility (Mulligan et al., 2001). However, copper bioavailability has shown to increase with increasing salinity (Forstner et al., 1989).

In order to determine metal speciation in soils, specific extractants are used to solubilize different phases of metals. A sequential extraction with solutions of increasing strengths provides a more precise evaluation of the different fractions of the metal (Rauret et al., 1999). Nevertheless, it should be mentioned that these measurements are operationally defined.

Plants also have an important role in metal availability in sediments, due for instance, to their ability to exude organic compounds that complex metals (Jones, 1998), but also because of their rhizosphere, which is a microenvironment in the surroundings of plants roots, that can be altered by the plants in relation to chemical composition (for example, causing changes in pH and redox potential) (Madureira et al., 1997). In addition plants can concentrate metals in their rhizosphere or in their tissues, influencing metals availability and mobility.
1.3. Phytoremediation

As seen before, the environment is threatened by a wide range of contaminants leading to the need of methods to treat and restore those polluted areas.

When it comes to remediate sediments contaminated by metals, two main approaches have been adopted: in situ and ex situ techniques. The first aims to increase the stabilization of metal on sediment particles, mainly by enhancing metal sorption, precipitation and complexation capacity on sediment reducing metals mobility but not decreasing its content, and the second consists in the extraction or separation of metals through several chemical, physical or biological methods from sediments that have been dredged from the river (Peng et al., 2009).

The methods used to remove metals from soil and sediments include techniques such as excavation and land fill, physical separation, thermal treatment, acid leaching, amendments, immobilization and electroreclamation (Jing et al., 2007; Mulligan et al., 2001).

However, these methods present various disadvantages like the modification of soil properties (such as sediment structure and fertility), disturbance of soil native microflora, the high costs of the processes and the intensive labor that they require (Ali et al., 2013).

An option to these techniques is phytoremediation, and according to Cunningham et al. (1995) is defined as the use of green plants to remove, contain, reduce the concentration or the toxic effects of environmental contaminants.

It is a technology with good cost-efficiency relation, it might be applied to vast areas, it does not require constant monitoring and maintenance and it is moved by solar power, characteristics that makes phytoremediation very well acceptable by the general public (Ali et al., 2013).

Phytoremediation is a technique that is applicable to both organic and inorganic contaminants, present in solid substrates (e.g. sediments), liquid
substrates (e.g. water), and the air, and is categorized into the following areas (Salt et al., 1998) and Fig. 5 illustrates these mechanisms:

- Phytoextraction: the removal of metals from soil by concentrating them in the harvestable parts of metal-accumulating plants;
- Phytodegradation: the use of plants and their associated microorganisms for the degradation of organic pollutants;
- Rhizofiltration: absorption and adsorption of metals by plants roots, from water and aqueous waste streams;
- Phytostabilization: reduction of contaminants bioavailability by plants roots;
- Phytovolatilization: the use of plants to volatilize pollutants;

Fig. 5. Mechanisms of phytoremediation involved in purifying contaminated soils and physiological processes that occur in plants during phytoremediation. Adapted from Lenart-Boroń & Boroń (2014).
Plants ability to uptake metals is beneficial for phytoremediation techniques, which can be useful to decontaminate polluted areas (Weis & Weis, 2004).

Some of the plants tolerance mechanisms against metals are: synthesis of metal binding peptides, vacuolar sequestration, immobilization of metals in cell walls, exclusion through the action of plasma membrane, phytovolatilization (Teixeira et al., 2014), playing an important role for the phytoremediation process.

The major strategies used for phytoremediation are phytostabilization, due to the ability of plants to capture metal in their rhizosphere, and phytoextraction, once metals are capable of uptaking metals.

Previous studies have shown the potential of several salt marsh plants to phytoremediate metals. For instance, in Almeida et al. (2008) and Almeida et al. (2009) was shown the potential of *H. portulacoides* to accumulate Cu in its roots and stems (although in less amounts).

When it comes to *P. australis*, its potential to phytoremediate metals, such as copper and cadmium, has been observed, and this plant was considered as able to phytoextract these metals (Almeida et al., 2011).

1.4. Bioaugmentation

A factor of success to the process of phytoremediation is the existence of microorganisms in the plant’s rhizosphere that can potentiate the ability of metal phytoremediation through symbiotic associations between plants and rhizobacteria (Jing et al., 2007).

Rhizobacteria possess characteristics that for instance, can allow them to change metals bioavailability through the release of chelating substances, acidification of microenvironment and changes on the redox potential of metals (Jing et al., 2007), which may influence metal uptake by the plants. In addition, rhizobacteria can improve metal uptake by plants by producing biosurfactants that enhance metals mobility (Lebeau et al., 2008).
On the other hand some rhizobacteria, the so called Plant Growth Promoting Rhizobacteria (PGPR), can increase plants biomass, leading to the removal of a higher amount of metal from the contaminated site. So, an increment in the number of these bacteria in the plants rhizosphere could improve phytoremediation.

Bioaugmentation is the augmentation of catabolically-relevant organisms to hasten remediation (Thompson et al., 2005). Although very developed to remediate organic contaminants from soils, it is applied in much lower extent to metals (Lebeau et al., 2008).

In most cases, the selection of the strains or microbial consortia to be used in bioaugmentation techniques does not take into account the microbial adaptation to the sites to which they are applied (Thompson et al., 2005). In fact, the environment of the contaminated site plays a determinant role in the survival and performance of the introduced microorganisms, and so, a promising approach to overcome these problems is known as autochthonous bioaugmentation, and consists in a bioaugmentation technology that only uses indigenous microorganisms from the contaminated site (Nikolopoulou et al., 2013).

However, autochthonous bioaugmentation for metal remediation is still little explored and its application to salt marsh is very scarce with only a study published by Nunes da Silva et al. (2014). This study has shown promising results, indicating that autochthonous bioaugmentation increased *P. australis* potential to phytoremediate cadmium contaminated sediments. So, this can be an important strategy to potentiate metal phytoremediation. However, different metals have different behaviors so it is important to test if this strategy will increase the phytoremediation potential that this salt marsh plant has being showing for other metals (e.g. Almeida et al., 2011).

1.5. Aims of the work

This work aims were to evaluate *P. australis* potential to phytoremediate copper contaminated sediment and to evaluate if rhizosphere microorganisms,
through autochthonous bioaugmentation, could potentiate phytoremediation by this salt marsh plant. A complementary aim consisted in the characterization of the microbial communities at the end of the experiment, as well as the characterization of microbial consortium used in the autochthonous bioaugmentation in terms of bacterial abundance and community structure.

In that sense, sediment vegetated with the plant *P. australis* was contaminated with copper and an enriched autochthonous microbial consortia resistant to the metal was added (Fig. 6). These consortia were prepared by exposing the sediments from the sampling site to copper. To clearly evaluate the role of the plant in the entire process, sediment non-vegetated was also tested.

Experiments were carried out in controlled conditions in a system that simulated the natural estuarine environment, namely the tidal regime, with sediments and plants with sediment attached to their roots collected from an estuary.

Fig. 6. Graphical abstract of the experiment, representing the interaction of rhizosphere microorganisms with plants. Adapted from Oliveira et al. (2014), available at: http://www.sciencedirect.com/science/article/pii/S0025326X14005906#.
CHAPTER II

Materials and methods

2.1. Experiments
   2.1.1. Sampling
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   2.2.6. Plants physiological parameters

2.3. Data analysis
2. Materials and methods

2.1. Experiments

2.1.1. Sampling

Specimens of the plant *P. australis* were collected, on 15\textsuperscript{th} April 2013, from the salt marsh area of the Lima River estuary (Viana do Castelo, Portugal, Fig. 7), at low tide.

The sampling site, located in the lower estuary, has an initial deep navigation channel and an upstream shallow saltmarsh zone and presents salinity ranging from 5 to 35 and turbidity from 1 to 15 NTU (Almeida et al., 2011).

The sampling consisted in collecting only green plants, with no senescent appearance and with similar size and age, with the sediment attached to its roots (cubes of approximately 20 cm side).

![Fig. 7. Lima river estuary (area of study) with the site of sampling site marked by the red arrow. Adapted from Almeida et al. (2011).](image-url)
Non-vegetated sediment cubes (similar dimensions) were also collected (sediment located within 0.5 m of the vegetated sediment). The cubes were placed in plastic vessels, in a total of 18 vessels (9 vegetated and 9 non-vegetated). These vessels had plastic taps and pebbles in the bottom (to minimize sediment loss through the taps) (Fig. 8).

![Fig. 8. Details on the collection of the plants and the sediment attached (1) and the vessels where both sediment with and without plants were placed (2).](image)

Individual plants were also collected into bags to evaluate copper initial levels and plants physiological parameters. Vegetated (rhizosediment) and non-vegetated sediment were collected to characterize the conditions of the sediments in the field (for microbial community and metal levels) and also for the development of the microbial consortia. For microbial consortia development estuarine water was also collected into plastic sterile bottles.

2.1.2. Microbial consortia development

Enriched copper-resistant autochthonous microbial consortia (Cu-RAMC) was developed using a process adapted from Lorah et al. (2008). In this procedure the original sediment and water slurry were enriched with microbial species resistant to copper through exposure to the contaminant and sequential dilutions that allow the survival of resistant organisms.

For each type of sediment (P. australis rhizosediment and non-vegetated sediment), 6 slurries were prepared with 60 mL of sediment, 120 mL of estuarine water collected from the sampling site and 3.5 mL of a 4 mol L⁻¹
glucose solution to promote the growth of the resistant microorganisms. To obtain the microbial consortia, to 3 of the 6 slurries of each type of sediment it was added 0.43 g of copper, being the other 3 slurries used as a control. Then, the slurries were incubated for 4 days at room temperature with agitation.

Therefore, 4 different microbial consortia were obtained: rhizosediment without and with copper addition, and sediment without and with addition of copper. Microbial consortia prepared without copper addition were used as controls for the experimental conditions used for the development of the consortia.

Each type of slurry was then mixed together, making approximately 360 mL, and divided to 3 culture flasks (ca. 100 mL each). To each new flask 100 mL of saline nutrient solution and 4 mL of the glucose solution were added.

After a new incubation of 4 days at room temperature with agitation, slurries were again mixed together (making approximately 600 mL) and transferred into 3 new culture flasks (100 mL each) and this last dilution was repeated 3 more times, being each time incubated for 4 days at room temperature with agitation. After that, each type of microbial consortia was mixed together to be used.

2.1.3. Microcosm experiments

The vessels were kept in a greenhouse placed outside the facilities of CIIMAR (Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal), exposed to environmental conditions of light and temperature. Vessels were randomly placed inside the greenhouse, to avoid influence of the greenhouse position (Fig. 9).
All vessels were irrigated through an automatic irrigation system, regulated to simulate the natural floods that occur in salt marshes (Fig. 10). Therefore, the vessels were submitted to two daily tidal cycles, each one having one period of draught and another of flood (with the duration of 6
Irrigation solution was a 1/4 strength Hoagland saline nutrient solution (with 10 salinity through addition of NaCl, being this the average salinity found in the estuary where the plants were collected). The solution was prepared when needed, by adding to 200 L of dechlorinated (through charcoal filter) tap water, 2 L of Hoagland solution and 2 Kg of sodium chloride. This solution was adapted from Hoagland & Arnon (1950). Vessels had an acclimation period of two weeks before the beginning of the experiments, during which the microbial consortia was developed (section 2.2). After this period, samples of sediment from the vessels were collected (ca. 5 g) to characterize the initial microbial community in the beginning of the experiment.

To 6 vessels with *P. australis* and 6 with non-vegetated sediment it was added a copper saline solution with a concentration chosen to result in a sediment copper concentration of 270 µg g⁻¹. This concentration corresponds to the ERM (effects range-median) value, which is the value of the sediment quality guideline that indicates the pollutant concentration above which it is frequent to occur adverse biological effects, in marine and estuarine sediments (Long *et al.*, 1995). This copper concentration may not be frequently found in estuaries but attending to the capacity of the plant to uptake metals, namely copper, this concentration was chosen to see a clear effect on the plant.

To half of these copper contaminated vessels, 3 with plant and 3 without plant, it was then added the respective microbial consortium.

Six vessels were kept without copper contamination (3 vessels with and 3 without plants) as control. Table 1 describes the treatments used in the experiment.
Table 1. Schematization of the experiment.

<table>
<thead>
<tr>
<th>Vessels (3 replicates per treatment)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vegetated:</strong></td>
<td><strong>Non vegetated:</strong></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Copper addition</td>
<td>Copper addition</td>
</tr>
<tr>
<td>Copper and bioaugmentation addition</td>
<td>Copper and bioaugmentation addition</td>
</tr>
</tbody>
</table>

All vessels were kept in the conditions described above for 2 months, being dismantled afterwards.

2.1.4. Vessels dismantling and samples preparation

For vessels dismantling, rhizosediment was carefully separated from the respective plant subterranean structures.

Rhizosediments and non-vegetated sediments were immediately collected. A portion was stored at -20 ºC for microbial community assessment. Another portion was put to dry at room temperature until constant weight for metal determinations.

Plants were washed with deionized water and divided into roots, rhizomes, stems and leaves. The different tissues were divided in two portions: one was dried at room temperature for metal quantification (Almeida et al., 2004) and the other was stored at -20 ºC for plants physiological parameters analysis (Ju et al., 2011).

For plants physiological parameters the sample preparation procedure was rapidly performed to prevent the oxidation of the samples. Although plants may have been in some stress throughout their handling, because cellular changes may occur immediately after harvesting the plant, all plants were treated similarly to allow comparison among them.
These procedures were used not only for the dismantled vessels but also for the samples collected at the sampling time for initial characterizations.

Sediments, rhizosediments and plants tissues dry weights were recorded.

2.2. Analytical determinations

2.2.1. Material and reagents

To prevent contamination, labware and sampling materials were soaked in a 20 % (v/v) HNO₃ solution for a minimum of 24 hours, rinsed repeatedly with bi-deionised water (conductivity < 0.1 mScm⁻¹) and dried in a Class 100 laminar flow hood.

The sample manipulation was carried out in a clean room with Class 100 filtered air.

All reagents used were pro analysis grade or equivalent.

2.2.2. Microbial abundance

Total Cell Counts (TCC) were estimated by the 4', 6'- diamidino-2-phenylindole (DAPI) direct count method (Kepner & Pratt, 1994; Porter & Feig, 1980), to monitor microbial abundance of the sediment and rhizosediment in the beginning and at the end of the experiment in all the different treatments.

For that, to each type of sediment (0.25 g of homogenized sediment) it was added 2.5 mL of dilution water, being afterwards fixed with formaldehyde (4% (v/v)). After that, it was added a 12.5 % (v/v) Tween solution and samples were stirred and sonicated for 10 min at low intensity.

Sub-samples of fixed sediment samples were then stained with 10 µL of DAPI, incubated for 12 min and filtered onto black Nucleopore polycarbonate filters (0.2 mm pore size, 25 mm diameter, Whatman, UK). Membranes were
set up in glass slides and cells counted on an epifluorescence microscope (Leica DM6000B) with a minimum count of 15 random microscope fields for each replicate.

To calculate the total number of bacteria was applied the following formula:

\[
\text{Total Number of Bacteria (cells per mililiter)} = N \times A_f \times d \times (A_g \times V_s)^{-1} \times 10^6
\]

In which:

\( N \) - number of cells counted

\( A_f \) - the effective area the area of the filter (mm\(^2\))

\( d \) – dilution factor (when performed)

\( A_g \) - the effective area the area of the counting grid (µm\(^2\))

\( V_s \) – volume of sample (mL)

\( 10^6 \) – conversion factor from mm\(^2\) to µm\(^2\)

2.2.3. Microbial community structure using Automated rRNA Intergenic Spacer Analysis (ARISA)

A modified CTAB extraction protocol (Barrett et al., 2006) was used to extract total DNA from the different sediments and DNA quality was evaluated through visualization on 1.5% agarose gels. For the microbial consortia developed, total DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories).

The bacterial community structure of the sediments and also of the microbial consortia was performed by automated rRNA intergenic spacer analysis (ARISA) and ITSF and ITSR eub primers set were used to amplify extracted DNA (Cardinale et al., 2004).
Polymerase chain reactions (PCRs), made in duplicate, were performed in 25 µL volumes containing 0.5 µL of sample, 10.5 µL of water, 0.4 µM of both primers, 0.2 mM dNTPs, 3x Taq PCR buffer, 2.5 U Taq DNA polymerase, 2 mM MgSO₄, and 1 mg/mL bovine serum albumin.

The PCR mixture was held at 94 ºC for 2 min for the initial denaturation, followed by 30 cycles of 94 ºC for 45 s, 55 ºC for 30 s, 72 ºC for 2 min, and a final extension at 72 ºC for 7 min.

The quality of the PCR products was estimated on 1.5 % agarose gel, and PCR replicates were combined and purified using the UltraClean 15 DNA Purification Kit (MO BIO Laboratories). Purified product was quantified using the Qubit dsDNA HS Assay Kit (Molecular Probes) and the Qubit fluorometer (Invitrogen). The sample fragments were run on a ABI3730 XL genetic analyzer at STABVIDA Sequencing Facilities (Lisbon, Portugal).

2.2.4. Metal determinations

To measure metal levels in sediments to ca. 0.25 g of dry sediment it was added 5 mL of concentrated HNO₃. To measure the metal levels in plant tissues, to ca. 0.50 g of dry plant sample it was added 5 mL of a 30 % H₂O₂ solution and 1 mL of concentrated HNO₃. Then, samples were digested in a high pressure microwave system (Ethos, Milestone) with Teflon vessels.

After this, levels of copper were determined by atomic absorption spectrophotometry with flame atomization (PU 9200X, Philips), following procedures validated before in the laboratory (Almeida et al., 2004).

2.2.5. Metal availability in sediments

Metal availability in sediments was determined using a sequential extraction protocol, according to the Measuring and Testing Program of the European Community (Rauret et al., 1999).
Portions of sediment were sequentially treated with: 0.11 M CH₃COOH solution (exchangeable fraction) and 0.5 M NH₂OH.HCl solution (fraction bound to Fe and Mn oxy-hydroxides), being the metal analyzed in solution as described in 2.2.4. The metal levels in the organic plus residual fraction was calculated by the difference between total metal levels and the sum of the metal levels in the fractions exchangeable and bound to Fe and Mn oxy-hydroxides.

Percentage of each fraction was calculated in relation to the total metal levels. More details can be found in Almeida et al. (2004).

2.2.6. Plants physiological parameters

Chlorophylls (a, b and total) and carotenoids were extracted and quantified in P. australis leaves according to a protocol adapted from Abadia et al. (1984). For that, in a 50 mL tube it was added ca. 0.5 g of P. australis leaves fresh material and 12.5 mL of a 0.4 % (w/v) calcium carbonate methanolic solution. After 48 h kept in the dark, 1 mL aliquot was diluted into 25 mL of de-ionized water and absorbances were recorded at 480 nm, 663 nm, 645 nm and 480 nm in an UV/Vis spectrophotometer.

The extracted amount (expressed in mmol g⁻¹) of total chlorophyll (Equation 1), chlorophyll a (Equation 2), chlorophyll b (Equation 3) and carotenoids (Equation 4) was calculated as follows. Each sample was analyzed in triplicate.

Equation 1:

\[
\text{Total Chlorophyll} = (8.02 \times A_{663} + 20.21 \times A_{645}) \times \frac{0.00125 \times 25}{\text{fresh weight (g)}}
\]

Equation 2:

\[
\text{Chlorophyll a} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times \frac{0.00125 \times 25}{\text{fresh weight (g)}}
\]
Equation 3:

\[ Chlorophyll\, b = (22.9 \times A_{645} - 4.68 \times A_{663}) \times \frac{0.00125 \times 25}{fresh\, weight\, (g)} \]

Equation 4:

\[ Carotenoids = \left( A_{480} + 0.114 \times A_{663} - \frac{0.638 \times Abs_{645}}{112.5} \right) \times \frac{0.00125 \times 25}{fresh\, weight\, (g)} \]

The total acid-soluble SH compounds (total thiols) were measured in roots and leaves of *P. australis* following the procedure of De Vos *et al.* (1992).

Plant tissues were flash-frozen in liquid nitrogen, tritiated and extracted with a 0.1 M HCl solution in a vortex. Afterwards, the solution was centrifuged for 30 min at 2000 rpm and filtered through a 0.45 µm cellulose nitrate membrane. Then, 600 µL of the extract (with acid pH) was mixed with 1.26 mL of K$_2$HPO$_4$ and 50 µL of a 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) solution.

The absorbance at 412 nm was measured 5 minutes after that and calibration was performed by using reduced glutathione (GSH) standard solutions (0 mg/L – 20 mg/L) prepared in a 0.1 M HCl solution.

2.3. Data analysis

Through a process previously validated by Mucha *et al.* (2013), a matrix of ARISA aligned fragments and fluorescence values was created and imported to the PRIMER 6 software package (version 6.1.13) (Clarke & Gorley, 2006). Data were normalized using the presence/absence pretreatment function and samples were then analyzed using the Bray-Curtis similarity method and clustered in the complete linkage mode with the default parameters (5% significance, mean number of permutations, 1000; number of simulations, 999), for generating a dendrogram based on percent similarity. A multidimensional scaling (MDS) plot was then generated using the default
parameters with a minimum stress of 0.01 to generate a configuration plot based on percent similarity.

For microbial analysis for each sample, three independent replicates were performed and mean values and respective standard deviations were calculated. The STATISTICA v.12 software (StatSoft, Tulsa, USA) was used to evaluate statistically significant differences among samples (through ANOVA tests) for the results obtained regarding the microbial community structure tests.

To assess the similarity of bacterial community composition among microcosms, an analysis of similarities (two-way crossed ANOSIM, based on Bray-Curtis similarity) was carried out. The ANOSIM is a permutation-based hypothesis statistical test, an analogue of the univariate ANOVA, which tests for differences among groups of (multivariate) samples from different locations or experimental treatments; the values of the R statistic were an absolute measure of how well the groups were separated and ranged between 0 (indistinguishable) and 1 (well separated).

The bacterial richness was estimated as the total number of unique OTUs (peaks) identified within each ARISA profile, assuming that the number of peaks represented the species number (phytotype/genotype richness).

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 plant tissues, due to the high natural variability, three replicates were analyzed for each vessel and then mean values and respective errors were calculated for each treatment. To evaluate statistically significant differences between treatments the students’ t-test (p<0.05) in GraphPad Prism 6 software was used.
CHAPTER III

Copper phytoremediation by a salt marsh plant (Phragmites australis) enhanced by autochthonous bioaugmentation

3.1. Results

3.1.1. Plants biomass

3.1.2. Physiological parameters

3.1.2.1. Chlorophylls

3.1.2.2. Total thiols compounds

3.1.3. Copper levels

3.1.3.1. Copper levels in P. australis tissues

3.1.3.2. Copper levels in sediment

3.1.3.3. Metal speciation

3.2. Discussion of results

3.3. Conclusions
3. Copper phytoremediation by a salt marsh plant (Phragmites australis) enhanced by autochthonous bioaugmentation

The present work is published in:


3.1. Results

3.1.1. Plants biomass

Total plants biomass weight varied among treatments, being the highest weight observed in the plants where no copper was added to their sediment (control). In fact, although differences were not statistically significant (Table 2), the total weight of the plants used as control was 12.5% and 40% higher than that of the plants exposed to sediment with copper addition without and with bioaugmentation, respectively. This difference was particularly noticeable in the plant roots and rhizomes biomass.
Table 2. Plants dry weight (mean and standard deviation, n = 3) per vessel and per area for different treatments (control, copper addition and copper addition plus bioaugmentation). In parenthesis are indicated the percentage of the tissues weight relatively to the total biomass weight. Different letters indicate significant differences (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Copper addition</th>
<th>Copper and bioaugmentation addition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total (g)</strong></td>
<td>104 ± 46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total (kg/m&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>3 ± 1</td>
<td>2.3 ± 0.4</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Roots (g)</td>
<td>9 ± 5&lt;sup&gt;a&lt;/sup&gt; (8%)</td>
<td>3 ± 2&lt;sup&gt;b&lt;/sup&gt; (3%)</td>
<td>1.2 ± 0.7&lt;sup&gt;b&lt;/sup&gt; (2%)</td>
</tr>
<tr>
<td>Rhizomes (g)</td>
<td>11 ± 6&lt;sup&gt;a&lt;/sup&gt; (10%)</td>
<td>6 ± 3&lt;sup&gt;b&lt;/sup&gt; (7%)</td>
<td>4.0 ± 0.7&lt;sup&gt;b&lt;/sup&gt; (7%)</td>
</tr>
<tr>
<td><strong>Belowground structures (g/m&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>243 ± 125</td>
<td>120 ± 66</td>
<td>65 ± 42</td>
</tr>
<tr>
<td>Stems (g)</td>
<td>62 ± 27&lt;sup&gt;a&lt;/sup&gt; (60%)</td>
<td>61 ± 10&lt;sup&gt;a&lt;/sup&gt; (68%)</td>
<td>46± 5&lt;sup&gt;a&lt;/sup&gt; (73%)</td>
</tr>
<tr>
<td>Leaves (g)</td>
<td>22 ± 9&lt;sup&gt;b&lt;/sup&gt; (22%)</td>
<td>20 ± 5&lt;sup&gt;a&lt;/sup&gt; (22%)</td>
<td>11 ± 3&lt;sup&gt;b&lt;/sup&gt; (18%)</td>
</tr>
<tr>
<td><strong>Aboveground structures (g/m&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>1056 ± 711</td>
<td>1019 ± 589</td>
<td>711 ± 477</td>
</tr>
</tbody>
</table>

Regarding aboveground structures, biomass was identical between control and copper addition treatment; however, it was lower when bioaugmentation was applied. The effect of copper addition on plant biomass was also evident in the biomass percentage relatively to total weight of each tissue.

In fact, in the treatment with copper, belowground structures accounted less for total plant biomass than in control. This indicates that probably copper affected the development of the roots and rhizomes of the plants. It should be mentioned that total biomass weight of plants in the field (in a cube of similar dimensions to the ones placed in vessels) was slightly lower (ca. 13% lower, although not significantly) than that in control vessels indicating that some
biomass was produced during the 2 months of experiment. In fact, although roots biomass was identical between field and control plant, aerial structures biomass increase ca. 25% during the experimental period.

In the plants exposed to sediment with copper addition a significant difference was observed between the ones with bioaugmentation and the ones without, as the plants to which it was added the microbial consortium presented 32% less total biomass weight relatively to the ones with no microbial consortium addition. However, the biomass percentages of the different tissues relatively to total weight were, in general, similar between these two treatments.

3.1.2. Physiological parameters

To assess possible stress symptoms, several physiological parameters, such as chlorophylls (chlorophylls a and b and Total chlorophylls), carotenoids and total thiols compound levels, were measured in leaves of the plants. It should be mentioned that these parameters were also measured in the plants collected in the field (Table 3), being the measured values in general higher than those measured in control plants (Table 3, Fig. 11, Fig. 12).

Table 3. Physiological parameters (chlorophylls (total chlorophylls, chlorophylls a and b), total carotenoids and total thiols compounds) measured in leaves and roots of the plants collected in the field.

<table>
<thead>
<tr>
<th>Chlorophylls (mmol chlorophyll/g)</th>
<th>Total chlorophylls</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Leaves</td>
<td>0.32 ± 0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total thiols compounds (mg GSH / g plant tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
</tr>
<tr>
<td>Leaves</td>
</tr>
</tbody>
</table>
This indicates that the plants suffered an adaption to the experimental conditions over the two months. Therefore, the effects on plants due to the addition of copper were evaluated by comparison between control and treatments with copper.

3.1.2.1. Chlorophylls

Fig. 11 presents the results related to the amounts of chlorophylls and carotenoids measured in leaves of the plants of all treatments.

![Bar chart showing concentrations of chlorophylls and carotenoids](image)

**Fig. 11.** Concentrations of chlorophylls (Total chlorophylls, chlorophylls a and b) and carotenoids in leaves of the plants subject to sediment without (control) or with addition of copper, the later without or with bioaugmentation application. Different letters indicate a significantly different value (p<0.05).

When comparing the treatments with copper with the control, no significant differences were observed in the chlorophylls concentrations, although a tendency to increase can be seen for total chlorophylls and chlorophyll b levels.
So, the exposure to sediment contaminated with copper did not influence significantly the levels of these compounds in the plants leaves. Regarding carotenoids, however, there was a significant increase in their levels when the plants were exposed to sediment with copper comparatively to control. This indicates that the metal affected the levels of these compounds inducing probably their production in the plants leaves as a mechanism of defense against copper toxicity.

On the other hand, no significant differences were observed in the levels of chlorophylls or carotenoids between the plants exposed to sediment with and without addition of the microbial consortium.

3.1.2.2. Total thiols compounds

Regarding total thiols compounds levels in *P. australis* roots it was observed a significant increase in both treatments with copper in relation to the control, being the concentrations ca. 4 times higher (Fig. 12.A).

![Fig. 12. Concentrations of Total thiols in roots (A) and leaves (B) of the plants subject to sediment without (control) or with addition of copper, the later without or with bioaugmentation application. Different letters indicate significant differences (p<0.05).](image)

The same tendency to increase total thiols compounds levels in leaves was observed among treatments with copper when comparing to the control, although values were significantly different only between control and the
treatment with bioaugmentation (Fig. 12.B). But for leaves, that tendency was also observed between both copper treatments, without and with bioaugmentation application, with higher values being found in leaves of plants exposed to sediment with addition of the consortium with Cu resistant microorganisms.

3.1.3. Copper levels

3.1.3.1. Copper levels in *P. australis* tissues

Copper levels in the different tissues of the plants of control were approximately half of the ones observed in the tissues of plants in the field.

This decrease demonstrates the adaptation of *P. australis* to the experimental conditions, over the two months. To evaluate copper uptake by the plants, copper levels in plant exposed to medium with added copper were compared with those of plants in control vessels (without copper addition, but subject to the same experimental conditions).

![Graph showing copper concentrations in different plant tissues](image)

*Fig. 13. Copper concentrations in roots, rhizomes, stems and leaves of the plants subject to sediment without (control) or with addition of copper, the later without or with bioaugmentation application. Different letters indicate significant differences (p<0.05).*
Comparing the systems with addition of copper with the control, it is clear that an increase of the metal levels in the medium lead to an accumulation of copper in the belowground structures and also in the stems, indicating metal translocation. However, it was observed a higher accumulation of copper in the belowground structures of *P. australis* (roots and rhizomes) compared to the aboveground structures (stems and leaves) (Fig. 13), showing the potential of this plant as a phytostabilizer.

When it comes to metal uptake by plant, significant differences between the treatments with and without microbial consortium resistant to copper addition were only found for stems of *P. australis*. In fact, in stems the addition of the microbial consortium led to a significant increase of metal concentration (ca. 2 times higher) in comparison with the treatment with only copper addition. Therefore, it is possible to establish a correlation between addition of microbial consortia resistant to copper and its translocation to plants stems.

Fig. 14 presents the total Cu in each structure of the plants with the different treatments, calculated attending to the plant structures biomasses. In this case, the same increase in the amount of Cu in stems when the microbial
consortium was added was also observed. On the other hand, there was a tendency for the total amount of copper to decrease in the belowground structures when the microbial consortium was added (only significant for rhizomes).

3.1.3.2. Copper levels in sediments

When comparing vegetated sediment with non-vegetated sediment, it can be clearly seen that the presence of the plant in the sediment contributed to a higher retention of the metal in its rhizosphere (Fig. 15), showing therefore, the potential of phytoremediation, in specific the ability of the plant to immobilize the metal in the rhizosphere.

![Copper concentrations in sediment](image)

**Fig. 15.** Copper concentrations in sediment without (control) or with addition of copper, the later without or with bioaugmentation application. Results are shown for both vegetated and non-vegetated sediments. For each treatment, different letters indicate significant differences (p<0.05). The initial sediment presented a copper concentration of 35 ± 2 µg Cu/g sample.

No significant differences were observed regarding the addition of the microbial consortium to the sediment, although values showed a tendency to decrease in the case of the vegetated sediments.
3.1.3.3. Metal speciation

In relation to the metal speciation, in both vegetated and non-vegetated sediment it was observed an increase of total bioavailable copper when microbial consortium was added (Fig. 16). In fact, the metal bound to organic matter and residual (the less bioavailable fraction) showed a decrease in vegetated and non-vegetated sediment of 10% and 7%, respectively when bioaugmentation was applied.

3.2. Discussion

It has been shown that metal phytoremediation by different plants can be enhanced through the inoculation of metal resistant microorganisms in the plants rhizosphere (Azcón et al., 2009; Shao et al., 2010). Although in most of these studies specific bacterial strains were used, the use of microbial consortia may be more successful because it provides higher metabolic diversity. In addition, most studies used exogenous bacterial strains inoculation (Ma et al., 2011), whereas studies using indigenous microbes are
scarce, with only a few examples for specific autochthonous bacterial strains (e.g. Azcón et al., 2009; Aboushanab et al., 2006). Moreover, these inoculations were carried out in the rhizosphere of soil plants to recover metal contaminated soils and studies with wetlands plants are to our knowledge almost inexistent. In fact, only two studies have shown that rhizobacteria could be important in the accumulation and attenuation of toxicity of Hg to saltmarsh plants (de Souza et al., 1999; Caslake et al., 2006). But very recently a study by the authors showed that autochthonous bioaugmentation with Cd resistant microorganisms could be useful for the enhancement of P. australis phytoremediation potential for Cd contaminated estuarine sediments (Nunes da Silva et al., 2014). In fact, after inoculation, a higher (up to 7 times) metal translocation within the plant was observed, being Cd a non-essential metal. However, interactions between plants and microorganisms will depend also on the metal and it is important to test if this strategy of autochthonous bioaugmentation will be successful for other metals, namely with essential metals like Cu.

So, in the present study a copper resistant autochthonous microbial consortium was inoculated in the rhizosphere of P. australis to evaluate if it could enhance the Cu phytoremediation potential already observed for this plant (e.g. Almeida et al., 2011).

After 2 months of exposure, the plant accumulated significant amount of the metal, being higher copper concentrations found in the belowground structures (roots and rhizomes) of P. australis. Previous studies carried out in the field have shown also significantly higher amounts of copper in belowground structures of P. australis than in its aboveground tissues (Windham et al., 2003; Almeida et al., 2011; Weis et al., 2002; Salem et al., 2014; Ali et al., 2002). So, present results confirm the potential of this plant as a phytostabilizer, the plant being able to accumulate copper in its roots and rhizomes after metal retention in the surrounding environment. In fact, metal levels in vegetated sediments after 2 months were significantly higher (2 to 3 times) than those in non-vegetated sediments, a feature previously observed (e.g. Almeida et al., 2004). This might be related to the ability of the plant to retain metal in its rhizosphere through reduction of leaching processes, but
also to the capacity of the plant to oxidize rhizosphere sediment resulting in the remobilization of the metal (Weis & Weis, 2004).

However, no significant differences were found between the treatments with and without addition of the microbial consortium resistant to copper regarding metal amounts in belowground structures. So, autochthonous bioaugmentation did not increase the phytostabilization potential of the plant. A similar result was observed when the same strategy was applied to recover Cd contaminated sediments with this plant (Nunes da Silva et al., 2014).

Although roots are very efficient in restricting the flow to the vascular tissue and therefore, in controlling the amounts of metal transported to the higher structures (Weis & Weis, 2004), in the present study, after 2 months, translocation of the metal also occurred. Nevertheless, copper concentrations were still lower than in the belowground structures, pointing out to the low capacity to translocate metal into stems and leaves.

The low translocation factor might result as a defense from the phytotoxic effects of metals like Cu (Salem et al., 2014), protecting aboveground tissues of high metal concentrations.

Copper is an essential metal for plants, being involved in the normal growth, metabolism and development and its optimum levels in plant tissues are in the range of 1–5 µg g⁻¹. However, it have been shown that higher copper concentrations (up to 30 µg g⁻¹) have the ability to induce negative effects, such as the generation of reactive oxygen species (ROS) in plants (Anjum et al., 2012).

In the present study, as mentioned, metal translocation occurred, although it was only significant for stems, as copper levels in leaves displayed no significant differences between plants exposed to sediment with and without addition of copper. The levels of copper in stems of plants to which copper was added were in the range of 13 and 23 µg g⁻¹, values much higher than the upper limit of the optimal concentration considered for copper.

Although no visual toxicity in aboveground tissues was observed, physiological parameters analysis in leaves indicated that the plant might have been in stress due to the presence of copper in its tissues. For instance,
although chlorophylls levels were identical, an increase in the production of thiolic compounds and carotenoids when copper was added to the sediment was observed, which could indicate a response to the oxidative stress caused by the uptake of copper by the plant stems. Plants are subjected to diverse environmental conditions, which by their own are able to cause stress to the plants due to their variations. However, with the increasingly contamination that salt marsh areas are target, metals are an additional disturber factor for salt marsh plants, inducing oxidative stress. So, thiolic compounds and carotenoids might be related to the defense of the plant against ROS, caused namely by metals such as copper, once copper is able to produce ROS (Schützendübel & Polle, 2002). In fact, carotenoids are efficient antioxidants that scavenge singlet molecular oxygen and peroxyl radicals (Stahl & Sies, 2003). Regarding thiolic compounds, cysteine, for instance, intervenes directly in the reduction of ROS, also complexing metals (Hernandez-Allica et al., 2006). Another thiolic compound glutathione reduces and scavenges ROS and acts as detoxifying agent towards pollutants, such as metals, being a precursor for phytochelatins, which are also very important against metal toxicity (Anjum et al., 2012).

Metal translocation in the plant increased when the microbial consortium resistant to copper was added to the sediment. In fact, copper concentration in plant stems increased up to two times relatively to plants exposed to sediment without inoculation. In addition, comparatively to control (plants exposed to sediment without addition of copper) there was also a significant increase in copper concentrations in plant leaves. So, the microbial consortium increased P. australis potential to phytoextract copper from contaminated sediments. A similar behavior was observed for this plant when the same strategy was applied to recover Cd contaminated sediments (Nunes da Silva et al., 2014), indicating that this strategy seems to work for both metals despite their differences.

Microorganisms associated to the plant can potentially improve metal uptake by altering metals solubility, availability and transport through soil pH reduction, chelators releasing, phosphorus solubilisation, or redox potential changing (Ma et al., 2011). For instance, microorganisms can produce iron
chelating siderophores, which are able to solubilize unavailable forms of metals by complexation reactions (Rajkumar et al., 2012).

In the present case the addition of autochthonous microbial consortia resistant to copper caused an increase of the copper bioavailable amount in the sediment, which probably caused the higher metal uptake. In the non-vegetated sediment, the bioavailable fraction of copper also increased with the addition of the respective microbial consortium. However, this increase was observed in lower extent, pointing out to the influence plants might have on the microbial communities that can develop in the rhizosphere. It is well known that plants exert an important influence and can shape the structure and composition of microbial communities by enhancing their activity by root exudates composition and quantity (Bais et al., 2006; Koranda et al., 2011). And in the case of salt marsh plants it was already observed that these plants have an effect on the microbial community structure (Mucha et al., 2011; Ribeiro et al., 2013).

In addition, rhizobacteria may not only increase metal availability but also enhance plant growth and tolerance to metals, having the ability to stimulate plant defense mechanisms against metal toxicity, allowing higher metal translocation within the plant (Lebeau et al., 2008; Ma et al., 2011).

In the present study, as already mentioned, some plant defense mechanisms against metal toxicity were triggered due to the higher amount of copper in aboveground structures. When bioaugmentation was applied and copper levels increased even more these mechanisms were also activated. In fact, in the leaves of plants exposed to sediment with addition of copper and of the microbial consortium, despite the fact that chlorophylls and carotenoids levels were identical to those in leaves of plants without bioaugmentation, the higher amount of copper in aboveground tissues induced an increase in the total thiols compounds levels. As already mentioned, copper is a redox active metal, and its autoxidation results in the production of ROS such as $O_2^-$ and then formation of $H_2O_2$ and $OH^-$ via Fenton-type reactions (Schützendübel & Polle, 2002). Thiolic compounds can be involved in plant defense mechanisms once they are able to reduce ROS caused by exposure to metals.
The production of these thiolic compounds was also stimulated in the plant roots. This stimulation was identical in the treatments with copper, either with or without addition of the microbial consortium, probably because copper uptake by the plant roots was also identical for these two treatments.

Despite these defense mechanisms being triggered by the plant, the addition of copper resistant microorganisms to the rhizosphere of *P. australis* did not enhance plant growth. In fact, total plant biomass weight was lower when copper was present, being even lower when the consortium was added, *i.e.*, when the metal was more bioavailable.

The decrease was more noticeable in the belowground structures, although stems biomass has also shown the tendency to decrease. Despite this, stems percentage in relation to total weight has remained similar to the other treatments. So, of the various ways that microorganisms can influence the process of phytoremediation, such as stimulating the plant growth, promoting resistance to the stress caused by contaminants or altering the availability of contaminants in the rhizosphere, in the present study, the likely role of the microbial consortium consisted in changing the speciation of copper in the sediment, resulting in a higher bioavailability.

Copper could have affected the belowground structures. For instance, Ali *et al.* (2002) recorded significant reductions of root length in *P. australis* due to copper toxicity, being reduction of root elongation the factor that most contributed to the decrease of plant biomass at high concentrations of the metal. In the present study, no measure of root length was performed; however, a decrease of it could explain the total biomass weight decrease.

The study performed by Ali *et al.* (2002) recorded significant decreases in growth parameters for *P. australis* at Cu concentrations of 78.7 µM and above. However, this study was performed in hydroponic conditions, which points to the lack of works that study the effects of copper in the growth of *P. australis* in simulated environmental conditions, especially in salt marsh areas.

When considering total copper amount per structures, a tendency to decrease copper amount in belowground structures is observed, being significant only for rhizomes.
Even though there was preferential accumulation in roots and rhizomes, several studies in the field observed that *P. australis* could also be used as a phytoextractor. In fact, despite the low concentrations of Cu in the aboveground structures per g of tissue, considering the abundant aerial biomass of this plant, it can accumulate significant amounts of metal in these structures. As seen in this work, aerial biomass account for more than 80% of total plant weight. Despite the decrease of biomass observed in stems when microbial consortium was added, the phytoextractor potential of the plant was verified, with total copper in stems being higher when microbial consortium was added. So, the phytoextraction potential of this plant was enhanced by autochthonous bioaugmentation, which points out to the importance of this technique to improve the phytoremediation process. Therefore, there is the need in the future to better understand how the microbial consortia is able to influence the process, namely by improving the metal translocation and bioavailability.

Although *P. australis* does not possesses salt glands like other species of salt marsh plants (e.g. *Spartina alterniflora*), the plant can excrete metal from aerial structures by leaching from leaves surface through transpiration and water loss (Burke *et al.*, 2000). In fact, after the time period of the experiment, copper levels in the leaves of plants exposed to not contaminated sediment (control) were lower than those initially present in field plants. In addition, decay of plant aboveground tissue enriched with metal, and the possibility of metal returning to the sediment, and entering the food chain through herbivores (Weis & Weis, 2004) has to be considered.

So, the use of *P. australis* as a phytoextractor must be followed by several precautions, and careful removal of the aboveground tissues has to be taken in consideration, once harvesting of plants in salt marshes can have an impact in the management of these important areas.
3.3. Conclusions

With this work, the potential of *P. australis* for phytoremediation of copper contaminated salt marsh sediments was demonstrated as well as the ability of autochthonous bioaugmentation to increase the phytoextraction potential of this plant.

In fact, copper translocation into plant stems was enhanced when a microbial consortium with copper resistant microorganisms was added to the copper contaminated sediment.

Although there was a decrease in the plant biomass weight, the increased translocation resulted in the possibility of a higher removal of copper from the contaminated sediment.
CHAPTER IV

Characterization of microbial communities after autochthonous bioaugmentation application for enhanced phytoremediation

4.1. Results
   4.1.1. Microbial consortia development
   4.1.2. Microcosm experiments

4.2. Discussion

4.3. Conclusions
4. Characterization of microbial communities after autochthonous bioaugmentation application for enhanced phytoremediation

4.1. Results

4.1.1. Microbial consortia development

The autochthonous microbial consortia resistant to copper was developed using the sediment collected at the sampling site, being exposed to the metal and followed by sequential dilutions that allowed the survival of the microorganisms resistant to the metal. In this work, 4 types of microbial consortia were developed: rhizosediment and non-vegetated sediment exposed to copper and rhizosediment and non-vegetated sediment without exposure to copper. Only the microbial consortia exposed to copper was used on the experiment; the ones without exposure to copper were used as a control to evaluate the effects of the process of bioaugmentation by itself on the original microbial communities.

Electropherograms were obtained for all the samples of the different treatments from the ARISA fingerprints. As expected, the process of bioaugmentation and the addition of copper, produced differences in the microbial communities of the consortium developed. Those differences are visible in Fig 17, in which it is observed a reduction in the number of peaks followed by an increase in the peaks height in the microbial consortia electropherograms in relation to the respective initial sediments.
Fig. 17. ARISA electropherograms of ITS amplicons amplified from the initial sediments (*P. australis* rhizosediment and non-vegetated sediment) and the respective microbial consortia.
These differences are also visible in Table 4, in which a reduction in bacterial richness and diversity is observed, particularly when comparing microbial community structure in the initial sediment with that observed in the consortia with copper addition. For the rhizosediment, the differences in bacterial richness and diversity are statistically significant between the initial rhizosediment and the microbial consortium with copper addition, and for the non-vegetated sediment these differences are statistically significant for the two microbial consortia developed.

In Fig. 17 there are also visible differences between rhizosediment and non-vegetated sediment, among the different treatments.

Table 4. Bacterial richness (operational taxonomic units - OTU) and diversity (mean and standard deviation, n=3) based on ARISA results in the initial sediments and the respective microbial consortia without and with copper addition. *statistically significant differences in relation to the respective initial sediments (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Initial Sediment</th>
<th>Microbial consortia control</th>
<th>Microbial consortia Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacterial richness</td>
<td>Bacterial diversity</td>
<td>Bacterial richness</td>
</tr>
<tr>
<td>Rhizosediment</td>
<td>84 ± 38</td>
<td>3.7 ± 0.4</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>Sediment</td>
<td>133 ± 25</td>
<td>4.2 ± 0.4</td>
<td>62 ± 16 *</td>
</tr>
</tbody>
</table>

Fig. 18 presents a hierarchical cluster for the different microbial consortia and for the initial sediments used to develop the consortia.

It is clear in Fig. 18 that samples replicates are grouped together, showing good replication procedure.

The initial rhizosediment and non-vegetated sediment were dissimilar but were the most similar to each other, with similarities of approximately 30%. When the initial sediments are compared to the microbial consortia used as control, significant differences are observed (similarity of ca 20%).

However, when comparing microbial communities in the initial sediments with those in the microbial consortia with copper addition, those differences were even higher (similarity of only ca 10%).
Within the same type of treatments (initial sediments, microbial consortium control and microbial consortium with copper addition) there are also visible differences between the two types of sediment, rhizosediment and non-vegetated sediment.

4.1.2. Microcosm experiments

The microcosm experiment was assembled to evaluate if an autochthonous microbial consortia resistant to copper would influence *P. australis* phytoremediation potential. In parallel it was also evaluated the effects of this consortia on the initially present microbial communities, after the two months.

The experiments were carried out in greenhouses and the conditions caused changes in the microbial communities, once the initial microbial communities.
Communities were only ca 20% similar to the ones in the control at the end of the experiment (Fig. 19).

Despite this, all samples at the end of the experiment were compared to the control that was under the same experimental conditions.

At the end of the experiment, several parameters were evaluated, including the bacterial richness and diversity as well as the microbial abundance of the microbial communities present.

In Fig. 20 are presented the results for the bacterial richness and diversity for the different treatments at the end of the experiment, in the different types of sediment (rhizosediment vegetated with *P. australis*, and non-vegetated sediment). No significant differences were observed for both parameters, when comparing the treatments with bioaugmentation and bioaugmentation plus copper with the control.
When it comes to the microbial abundance in the samples collected at the end of the experiment for the two types of sediment (rhizosediment and non-vegetated sediment), significant differences among them were not observed, even though there was a slight tendency for the values of microbial abundance in the non-vegetated sediment to be lower (Fig. 21).

Fig. 20. A) Bacterial richness (operational taxonomic units – OTU) and B) bacterial diversity (mean and standard deviation, n=3) based on ARISA results for samples of the different treatments at the end of the experiment, for the two types of sediment.

Fig. 21. Microbial abundance in the two types of sediments, with different treatments, at the end of the experiment.
The microbial consortia prepared for bioaugmentation presented abundance of ca. $8.14 \log_{10}$ cells ml$^{-1}$ for the consortium developed from rhizosediment and of ca. $8.21 \log_{10}$ cells ml$^{-1}$ for the consortium developed from non-vegetated sediment, which are values higher than the microbial abundances registered in sediments at the end of the experiment. Despite this, the microbial abundances in the end of the experiment were similar between control and treatments with copper.

Fig. 22 represents a multidimensional scaling (MDS) for the different treatments at the end of the experiments, showing the similarities and dissimilarities among the microbial communities and how the addition of copper and the process of bioaugmentation were able to modulate them.

The MDS plot for the microbial community structure of the samples at the end of the experiment clearly separates the samples accordingly to the sediment type, being this a determining factor, which means the samples of *P. australis* rhizosediment were more similar to each other than to the samples of non-vegetated sediment.

However, the addition of copper also showed an influence when it comes to modulate the microbial communities structure, even though its influence was lower than the sediment type. Within the different treatments for each type of sediment, the treatments with copper and copper plus bioaugmentation were more similar to each other than to the control. The difference from control was more noticeable for the non-vegetated sediment.
Fig. 22. A) Multidimensional scaling (MDS) ordination based on Bray–Curtis similarities in the presence/absence matrix obtained from ARISA fingerprints of microbial communities at the end of the experiment for the different sediments. The letter R (samples with color green) represents the three treatments (control, copper addition – Cu – and copper addition plus bioaugmentation – Cu + BA) for *P. australis* rhizosediment and the letter S (samples with color blue) represents the same three treatments for the non-vegetated sediment. Numbers represent replicate samples. B) Detail of the MDS for the rhizosediment treatments.
These results are supported by analysis of similarities (two-way crossed ANOSIM) (Table 5), in which the separation of the samples according to sediment type is stronger than the separation due to copper addition.

Table 5. Two-way crossed ANOSIM test for different sediments (P. australis rhizosediment and non-vegetated sediment), copper and bioaugmentation addition, based on ARISA results at the end of the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Statistic value (R)</th>
<th>Significance level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sediment type vs Copper addition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global test Sediment type</td>
<td>1</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Global test Copper addition</td>
<td>0.824</td>
<td>0.1 %</td>
</tr>
<tr>
<td><strong>Sediment type vs Bioaugmentation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global test Sediment type</td>
<td>0.764</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Global test Bioaugmentation</td>
<td>0.201</td>
<td>10.7 %</td>
</tr>
</tbody>
</table>

Regarding bioaugmentation there was no significant differences between microbial communities’ structures in the sediments with or without bioaugmentation.

4.2. Discussion

In the beginning of the experiment, samples of the initial sediments were exposed to the metal to prepare the microbial consortia resistant to copper. The microbial consortia developed were characterized in terms of microbial structure.

The hierarchical cluster analysis based on ARISA profiles revealed a clear separation between the microbial assemblages that inhabited the initial sediments and the microbial consortia developed with them, without copper addition (control consortia), meaning that the experimental conditions of bioaugmentation were able to cause changes in the microbial community structure.

However, these differences were even more pronounced between initial
sediment and the microbial consortia with copper addition, demonstrating that copper had a clear effect in shaping the microbial communities. In fact, the presence of copper lead to lower bacterial richness and diversity, which is probably the result of the selection of specific species resistant to the metal. In fact, metals have been associated with selective pressure resulting in microorganisms resistant to the metal (Sandrin & Maier, 2003), which demonstrates the ability of the microorganisms to adapt after toxic metal exposure (Liebert et al., 1991).

The evolution of the microbial community structure to metal resistant community has been reported in previous works (Becker et al., 2006 and references therein, Turpeinen et al., 2004).

In addition, there were also differences between vegetated and non-vegetated sediments (which were only ca 20% similar), demonstrating that the presence of plant can also influence the microorganisms present.

So, each type of sediment produced a different consortium enriched in copper resistant bacteria. Other works have demonstrated that the soil from which the consortia are enriched influences the microbial communities (Wu et al., 2013).

To understand the behavior of the microbial communities in the process of bioaugmentation and their role in improving copper phytoremediation, microbiological experiments were carried out in which the prepared microbial consortia were added to the respective sediment (rhizosediment or non-vegetated sediment).

At the end of the experiment, the microbial community structure was assessed for samples of the different treatments. The analysis of the microbial communities at the end of the experiment is of great importance, once it provides information about the evolution of their structure.

Microbial communities in sediments at the end of the experiment presented changes in relation to the initial sediment, demonstrating that the experimental conditions also caused changes in the microbial community structure.

But also in this case, the presence of copper was an important factor in shaping the microbial communities, whether there was addition of
bioaugmentation or not.

However, the effects of copper were lower than the effects of the plant in the experimental vessels, being this the most important factor. In fact, in previous studies the plant has shown to be a determinant factor in the shaping of the microbial communities structures; Teixeira et al. (2014) and Mucha et al. (2011) also verified that microbial communities were different between vegetated and non-vegetated sediments, whether there was addition of copper or not. With petroleum hydrocarbons it was reported a similar effect in altering the structure of the microbial communities, where the presence of plant and the plant species acted as determinant factor, overriding the effect of the different treatments tested (Ribeiro et al., 2013).

These shifts in the microbial community when the plant is present might be related to the ability of the plant to alter sediments characteristics, by oxidizing or acidifying the rhizosediment, among other processes (Mucha et al., 2008; Weis & Weis, 2004)

The presence of plant lead to more similar microbial communities between the controls and treatments with addition of copper, than in non-vegetated sediment, despite the higher metal levels observed for the rhizosediments. This might be related to a protective effect of the plant to the microorganisms towards metal effects. In fact, it is known that through the composition and quantity of roots exudates, plants can greatly influence and shape the structure and composition of the microbial assemblages, through symbiotic relations (Bais et al., 2006; Koranda et al., 2011).

Regarding the addition of the microbial consortia to the experimental vessels, no significant differences were observed in the microbial assemblages between sediments with and without bioaugmentation. This result is similar to that obtained by Teixeira et al. (2014) that followed a similar approach of autochthonous bioaugmentation to enhance salt marsh plants potential for phytoremediation of Cd contaminated sediment. Also in this case, no shift in the communities’ structure was observed, even though different metals have different behaviors.

For rhizosediment, communities structure were even more similar showing once again the protective role of the plant.
So, bioaugmentation did not contribute for a shift in microbial structure. Considering that microbial abundance was identical, this can mean that the process of bioaugmentation was able to improve the process of phytoremediation without altering the microbial community structure at long term.

When bioaugmentation was added to the sediment it was observed an increase in the amount of Cu bioavailable in the sediment, increment that, in the case of rhizosediments, was probably responsible for the higher uptake of copper by the plant and also for the lower weight biomass of the plant roots as discussed in Chapter 3. So, this increment in bioavailable copper might be also responsible for the shift in microbial communities' structures as these communities have shown to respond to the metal presence, as seen for the prepared microbial consortia.

The process of bioaugmentation is seen as an advantageous strategy to potentiate the process of phytoremediation by plants, however, it is still little developed, with few works in the field of metal remediation in salt marsh areas (Nunes da Silva et al., 2014; Teixeira et al., 2014).

4.3. Conclusions

Copper has demonstrated an effect in changing the structure of the microbial communities present in the microbial consortia developed, probably favoring the selection of specific species resistant to the metal, but maintain bacterial richness and diversity.

Plant had also shown an important role in shaping the microbial communities, revealing a protective role against the toxicity of the metal to the microorganisms.

The development of the microbial consortia has proven to be beneficial for the phytoremediation process by *P. australis* and lead to an increase in the copper uptake by the plant, as seen before in Chapter 3.
In general, this technique has shown potential to be applied in areas with metal contamination. However, more knowledge is still needed to improve and provide better understanding on the mechanisms involved.
CONCLUSIONS

With this work, P. australis potential for phytoremediation of copper contaminated salt marsh sediments was demonstrated as well as the ability of autochthonous bioaugmentation to increase this plant phytoextraction potential. In fact, copper translocation into plant stems was enhanced (up to two times, without significant visual toxicity signs) when a microbial consortium with copper resistant microorganisms was added to copper contaminated sediment.

Higher metal translocation was probably caused by the higher metal bioavailability in sediments observed after the addition of the microbial consortium resistant to copper to the vessels.

Copper has shown to be an important factor when it comes to shaping the microbial communities probably favoring the selection of specific species resistant to the metal. However, the presence of the plant showed a protective role against the toxicity of the metal to the microorganisms.

To our knowledge, this is one of the few works that has studied autochthonous bioaugmentation effects in phytoremediation processes. Although, more research is needed to better understand processes by which microorganisms potentiate phytoremediation, the present study clearly indicates that bioaugmentation can be a technique for remediation of contaminated areas.

So, phytoremediation combined with autochthonous bioaugmentation can be a key strategy for the recovery moderately impacted estuaries, contributing for an efficient risk management strategy of estuarine and coastal

As future perspectives, there is the intent to do more research regarding the identification of specific microbial species resistant to copper, as well as the mechanisms through which that resistance was obtained and the implications to the microbial community structure that it implies.


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


