Impact of Microplastics in Phytoremediation of Metals by Estuarine Plants

Edite Joana Manjate

M 2017

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS
Impact of Microplastics in Phytoremediation of Metals by Estuarine Plants

Dissertation for the Master degree in Environmental Toxicology and Contamination submitted to the Abel Salazar Biomedical Sciences’ Institute from the University of Porto.

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“Neste mundo, tudo tem a sua hora; cada coisa tem o seu tempo próprio. Há o tempo de nascer e o tempo de morrer, o tempo de plantar e o tempo de arrancar o que se plantou, o tempo de matar e o tempo de curar, o tempo de destruir e o tempo de construir (...).

Que proveito tira das suas fadigas aquele que trabalha? Eu vi a tarefa que Deus impôs aos filhos dos homens para que dela se ocupem. Todas as coisas que Deus fez, são boas a seu tempo.”

Eclesiastes 3, 1-11
Acknowledgments

First of all, I would like to express my sincere gratitude to my supervisor Dr. Cristina Marisa R. Almeida. Without her knowledge, support, patience and guidance this dissertation would not have been possible.

I also would like to thank to my co-supervisor Dr. Sandra Cristina da Costa e Silva Ramos. For her guidance, availability, support and kind advice throughout my research studies.

My sincere appreciation to Dr. Ana Paula Mucha, for her support and help me to elaborate my report for the subject of Project and during the initial phase of my laboratory work.

To Filipa Santos, Bruno Gonçalves e Leonor Ferreira for their help in collecting the plants and preparation of elutriate solution for Cd experiments and to Alexandre Gouveia e Ana Silva for their help in Cd determinations.

To ALL my colleagues and superiors from ICBAS, FCUP and CIIMAR.

At last, but by no means the least, I would like to give my most heartfelt thanks and appreciation to my family. Thank you for being always there for me, for giving me motivation in good and bad moments and, most of all, for making my world a better place.

To all my friends, thank you for your understanding and encouragement. I cannot list all the names but I have you always present in my heart.

This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020 and by the structured Program of R&D&I INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources, reference NORTE-01-0145-FEDER-000035, namely within the Research Line ECOSERVICES (Assessing the environmental quality, vulnerability and risks for the sustainable management of the NW coast natural resources and ecosystem services in a changing world) within the R&D Institution CIIMAR (Interdisciplinary Centre of Marine and Environmental Research), supported by the Northern Regional Operational Programme (NORTE2020), through the European Regional Development Fund (ERDF).
Abstract

Microplastics (MP) (plastic debris < 5 mm) are emergent contaminants that are widely spread in natural systems, although their real impact in the environment remains understudied, mainly interferences in major ecological processes. The present work focused on estuaries areas since, to date, investigations related with the presence of MP in freshwater ecosystems are scarce.

Estuaries are among the most globally productive ecosystems, yet they also endure large anthropogenic pressures. Estuarine sediments are often contaminated with heavy metals and organic contaminants from runoff and human activities. Some salt marsh plants, like Phragmites australis, have the ability to recover contaminated soils through a process denominate phytoremediation. However, the presence of pollutants in the environment, as MP that potentially accumulate metals and other contaminants in their surface, may interfere with this ecological function by preventing the removal of contaminants in sediments through phytoremediation.

The aim of this work was to study the impact of MP in phytoremediation of sediments contaminated with trace metals, Cu and Cd, through the salt marsh plant P. australis. An additional aim was to investigate if the parameters type, aging, MP concentration and salinity affect MP capacity to sorb metals.

To achieve the proposed objectives, first preliminary tests with elutriate was carried out to study the interactions of MP with metals. Afterwards, salt marsh plants (Phragmites australis) collected from Lima estuarine area (NW Portugal) were exposed to elutriate solution doped with metal (Cu or Cd) or with metal plus polyethylene microspheres (PE) (Cu+PE or Cd+PE) in the presence or in the absence of sediments. After seven days of incubation, metal content on plants tissues and medium (sediments when present and elutriate solutions) were measured.

The results of preliminary test showed that metals were adsorbed only by film MP.

Plants accumulated substantial amounts of both metals, Cu and Cd, in their belowground tissues for all the treatments (metal and metal+PE, in absence and presence of sediments). The present study showed that selected MPs did not
have relevant effect on the capacity of plants to phytoremediate Cu and Cd contaminated media, although future research should investigate the effect other type of MP, namely film, since they were the only MP proved to adsorbed metals.
Resumo

Microplásticos (MP) (partículas plástico <5 mm) são contaminantes emergentes que se encontram amplamente espalhados nos sistemas naturais. No entanto, o seu impacto real no ambiente ainda é pouco conhecido, principalmente a nível da sua interferência nos principais processos ecológicos. O presente trabalho focou-se nas áreas estuarinas uma vez que, até à data, o número de investigações relacionadas com a presença de MP nos ecossistemas de água doce ainda é muito reduzido.

Estuários estão entre os ecossistemas mais produtivos a nível global, contudo, estão expostos a grandes pressões antropogénicas. Os sedimentos estuarinos são muitas vezes contaminados com metais e contaminantes orgânicos provenientes de actividades humanas e do escoamento de terra provocado pelas chuvas. Algumas plantas de sapal, como a Phragmites australis, têm a capacidade de recuperar solos contaminados através de um processo designado fitorremediação. No entanto, a presença de poluentes no meio ambiente, como os MP poderá, potencialmente, interferir com esta função ecológica, uma vez que estes têm a capacidade de acumular metais e outros contaminantes à sua superfície retendo-os nos solos. Desta forma os MP poderão impedir a remoção de contaminantes através da fitorremediação.

O objectivo deste trabalho foi estudar a influência dos MP na capacidade de plantas estuarinas, Phragmites australis, para fitorremediar metais, nomeadamente cobre e cádmio, presentes em sedimentos estuarinos. Estabeleceu-se como objectivo adicional investigar se os parâmetros tipo, idade, concentração de MP e salinidade podem afectar a capacidade de MP para absorver metais.

Para atingir os objectivos propostos, procedeu-se à realização de testes preliminares com elutriado, de modo a estudar as interacções entre os MP e os metais. Seguidamente, as plantas de sapal (Phragmites australis), recolhidas na área do estuário do Lima (NW Portugal), foram expostas a soluções de elutriado dopadas com metais (Cu ou Cd) ou com microesferas de polietileno (PE) (Cu + PE ou Cd + PE), na presença ou na ausência de sedimentos. Após sete dias de incubação mediu-se a concentração de metais presente nos tecidos das plantas e no meio experimental (nos sedimentos quando presentes e nas soluções de elutriado).
Através dos resultados dos testes preliminares inferiu-se que os metais foram absorvidos apenas pelas películas de MP.

As plantas acumularam quantidades substanciais de ambos os metais, Cu e Cd, nos seus tecidos subterrâneos em todos os tratamentos aplicados (metal e de metal + PE, na ausência e presença de sedimentos). O presente estudo mostrou que, nas condições testadas a presença de MP de PE no meio não teve um efeito relevante sobre a capacidade das plantas de fitorremediar Cu e Cd, contudo trabalhos futuros deverão investigar o efeito de outros tipos de MP, nomeadamente as películas que foram os únicos MP com capacidade de adsorver metais.
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Table 1: Cd concentrations (mean and standard deviation (in parenthesis, n=3)) in plants’ aboveground tissues (stems and leaves) after 7 days of exposure to elutriate solutions amended with Cd (1 mg/L) or Cd+PE (1 mg/L and 1500mg/L, respectively; PE – polyethylene microspheres), in presence or absence of sediments (Sed). Limit of detection (LD) = 0.7 µg/g)……………………………………………………………………………………………………………………………………37
List of abbreviations

Cd – cadmium
Cu – cooper
Fibers – fishing line fibers
Film – microplastics of film plastic bags
HD/LD-PE – high/low-density polyethylene
MP – microplastics
PAHS – polycyclic aromatic hydrocarbons
PE – polyethylene
PET – polyethylene terephthalate
POPs – persistent organic pollutants
PP – polypropylene
PS – polystyrene
PVC – polyvinyl chloride
PHC – petroleum hydrocarbons
PCBs – polychlorinated biphenyls
Zn – zinc
1. Introduction

1.1. Microplastics

1.2. Phytoremediation

1.3. Objectives
1. Introduction

One of today’s biggest concerns is the increasing waste of plastic. The main problem associated with the excessive utilization of plastics is the difficulty to develop a suitable infrastructure of waste management for this type of garbage (EU Commission, 2011). Other issue that has being raised is the formation and spread of microplastics (MP) in the environment.

MP are plastic debris with a diameter smaller than 5 mm. They can be intentionally produced for industrial purposes, as ingredients for cosmetics, such as, whitening toothbrush, face scrubs, body washes, or as resin pellets. Moreover, MP can be a product of larger plastics debris degradation (Barnes et al., 2009; EU Commission, 2011; Wagner et al., 2014).

To date, studies focused on the presence of MP in freshwater ecosystems are scarce, although there are clear evidences that this material is broadly spread along lakes, rivers and transitory systems as estuaries (Frias et al., 2010; Eerk es-Medrano et al., 2015; EU Commission, 2011). The main studies concentrate on saltwaters.

Plastic is an inert material, although, environmental dynamic can induce modifications on its surface and makes it suitable to adsorb pollutants (Holmes et al., 2014; Brennecke et al., 2016; Koelmans et al., 2016). In fact, there are evidences that MPs have capacity to accumulate contaminants in their surface, such as persistent organic pollutants, xenoestrogens and metals (Mato et al., 2001; Frias et al., 2010; Bakir et al., 2014; Holmes et al., 2014; Koelmans et al., 2016). Additionally, initial studies demonstrated that aquatic species could ingest MP, confounding them with food impacting their physiological fitness and also expose the organisms to contaminants present in particles surface (Browne et al., 2008; Oliveira et al., 2013; Wagner et al., 2014). These microparticles can enter in the organisms in a direct way, by ingestion, or indirectly through biomagnification, a process in which micromaterials are transferred along the food chain to higher trophic levels, including birds and mammals (Farrell & Nelson, 2013; Eerkes-Medrano et al., 2015; Avio et al., 2016).

The interaction of MPs with vegetable organisms is still a subject poorly investigated, although there are evidences that MPs may have an important impact on ecological functions. Recently, Bhattacharya et al. (2010) demonstrated
that in laboratorial conditions, freshwater Chlorella and freshwater/saltwater Scenedesmus algae absorb nanoparticles of polystyrene, leading to a decrease of their photosynthetic activity as consequence. Contrary, Davarpanah & Guilhermino (2015) did not observe an interference of polyethelene microspheres on the average specific growth rate of Tetraselmis chuii. Thus, it is important to investigate the impact of MP on vegetables organisms, namely on important functions as the phytoremediation.

Phytoremediation is an ecological technic that resorts to natural functions of plants to recover soil, sediment, surface water and groundwater environments contaminated with toxic metals, organics and radionuclides (Cunningham and Berti, 1993; Pradhan et al., 1998; Ali et al., 2013). Metals, contrarily from organic substances, are non-biodegradable, being persistent in the environment. The ecological contamination by metals has been increasing along the years, which can have negative impacts on environmental and human health (Ali et al., 2013). Studies on application of phytoremediation to remove metal and metalloids from soils or sediments have demonstrated promising results (Cunningham and Berti, 1993; Ali et al., 2013; Anjum et al., 2014; Silva et al., 2014). For instance, several salt marsh plants have shown capacity to accumulate metals from contaminated sediments contributing for their recovering (Almeida et al., 2011; Rocha et al., 2014; Ali et al., 2013; Silva et al., 2014). Several studies verified that Phragmites australis has the capacity of accumulate copper (Cu) and cadmium (Cd) in its belowground and aboveground tissues (e.g. Almeida et al., 2011; Silva et al., 2014; Oliveira et al., 2015). However, MPs ability to accumulate metals may prevent these pollutants for being removed from the substrate by plants, since such association will reduce the bioavailability of metals in soil (Ali et al., 2013). Further, due to its reduced size, MPs can possibly block the entrance of metals onto plant roots, decreasing metals accumulation by plants, affecting, therefore, plants phytoremediation potential. These facts can possibly increase the negative impact of MPs in the environment.

The aim of this work was to investigate possible interferences of MP in phytoremediation of sediments contaminated with trace metals (Cu and Cd) by the salt marsh plant P. australis. Moreover, it was also studied if physical properties (type and aging) and concentration of MP affect their capacity to sorb metals.
1.1. Microplastics

Classification

Actually, there is no standardized classification for plastics debris present in the environment. In the literature, it is common to find plastics divided by size, origin, shape, polymer type and colour (Wagner et al., 2014; Eerkes-Medrano et al., 2015). Wagner et al. (2014) also proposed some classifications groups based on size, origin type of polymer and shape:

- Size: plastics are divided in macroplastics (>25 mm), mesoplastics (5 to 25 mm), large MPs (1 to 5 mm), small MPs (20 µm to 1 mm) and nanoplastics (particles smaller than 20 µm);

MP are characterized as the following classification:

- Origin: MPs are defined as primary or secondary according to their origin. Primary MPs are industrially produced, for example as resin pellets, which are used as raw materials for plastics products, or as additives for personal care products, such as shower gels and peelings (EU Commission, 2011, 2013; Wagner et al., 2014). Secondary MPs results from degradation of larger plastics items, through natural processes of ageing as physical abrasion to smaller fragments or friction of plastics surface by UV radiation;
- Type of polymer: the most common polymers found in the environment are high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC). Accordingly, these are the most globally produced;
- Shape: MPs can be described as being fragments (with a rounded or angular shape), pellets (cylinders, disks or spherules), filaments (fibers) or granules.

Presence of MP in aquatic environments

Plastic is a material widely used due to its advantageous characteristics, namely, high persistence, resistance to corrosion and low electric and thermal conduction. However, these properties facilitate the accumulation of plastic debris in different types of ecosystems, for example surface waters, water column, shorelines and sediments, turning plastic harmful for the environment (Bockhorn et al., 1999, Barnes et al., 2009; EU Commission, 2011; Wagner et al.,
MPs have very heterogeneous distributions along the oceans, having a higher concentration in regions close to industrial centres or metropolitan areas, in enclosed and semi-enclosed seas such Mediterranean Sea (Barnes et al., 2009; Browne et al., 2011; GESAMP, 2015; Duis & Coors, 2016). Regarding the vertical distribution, water surface usually presents a higher concentration of MP comparing with water column (EU Commision, 2011; Duis & Coors, 2016). Additionally, MPs concentration tend to be higher in sediments than in water column or surface layer (Browne et al., 2011; Wagner et al., 2014; Duis & Coors, 2016).

To date, studies focused on the presence of MP in freshwater ecosystems are still scarce, although there are clear evidences that this material is broadly spread along lakes, rivers and transitory systems, as estuaries (Frias et al., 2010; EU Comission, 2011). Eerkes-Medrano et al. (2015) in their review studies reported the presence of MP in freshwater environments during the last years, namely:

- North America: Moore et al. (2011) found MP in the Los Angeles basin and affirmed that rivers transport large amounts of MP; Hoellein et al. (2014) detected microparticles of plastic in the North Shore Channel of Chicago, Castañeda et al. (2014) in the St. Lawrence River, and Zbyszewski & Corcoran (2011), Zbyszewski et al. (2014) and Eriksen et al. (2013) in the Great Lakes;
- Asia: Free et al. (2014) found MP in Lake Hovsgol in Mongolia.

These studies reported the presence of MP with distinct origins, sizes and compositions in freshwater ecosystem in different parts of the world (Eerkes-Medrano et al., 2015). In general, these studies also concluded that there is a link between the types of MP detected and the anthropogenic activities in the surround areas. The differences between freshwater and marine conditions on
degradation processes remain unknown. However, comparing these systems it is expected to found different degrees of physical forces, such as storms and wave action, and also, freshwater MP are expected to be expose to a more strong chemical degradation (Andrady, 2011). Free et al. (2014) suggested that lake conditions could expose MP to high levels of weathering owing to the relative larger penetration of UV light. Moreover, Brandon & Ohman (2016) concluded that UV radiation accelerate the oxidative degradation of MP. Finally, MP in estuaries are also subject to tidal cycle, and freshwater and marine MP can present similar degradation patters in their surface (Eerkes-Medrano et al., 2015).

### Source of MP

Studies suggested that the types of primary MP reaching aquatic systems include polyethylene, polypropylene and polystyrene particles present in cleaning and cosmetic products, through household sewage discharge, (Gregory, 1996; Eerkes-Medrano et al., 2015; GESAMP, 2015). MP of industrial origin comprise other source of primary MP in the environment, derived from spillage of plastic resin powders or pellets used for airblasting, for example (Gregory, 1996).

Regarding secondary MP, the breakdown from larger items can occur after plastics reach the environment due to exposure to environmental dynamics (Andrady, 2011; Eerkes-Medrano et al., 2015) or before, for example synthetic fibres from the washing of clothes (Browne et al., 2011). These fibres are predominantly made of polyester, acrylic and polyamide and normally are dominant at sewage disposal sites, having a long residence time both in the source areas as in freshwater systems (Zubris & Richards, 2005; Browne et al., 2011).

### MP as carriers of other contaminants

There are evidences that MP have capacity to accumulate contaminants and persistent, bioaccumulative and toxic compounds in their surface, such as persistent organic pollutants (POPs), xenoestrogens and metals (Mato et al., 2001; Frias et al., 2010; Bakir et al., 2014; Holmes et al., 2014).

Such accumulation may vary with the physical-chemical characteristics of the environment as salinity. Although Bakir et al. (2014) demonstrated that
salinity had no influence in the sorption rate of POPs by microplastics. An increase of salinity reduces POPs solubility, making them available to interact with sediments and buried plastics materials (Bakir et al., 2014).

Other factor that may influence the sorption of contaminants is the stage of degradation observed in the microparticles. Plastic is an inert material. However, environmental dynamic induces modifications on its surface and make it suitable to adsorb pollutants (Ashton et al., 2010; Holmes et al., 2014; GESAMP, 2015). Moreover, rapid degradation due to mechanical, thermal, chemical and photo oxidative stress processes can lead to loss of weight of the polymers (Pérez et al., 2010). This fact may cause alterations on the properties of MP and possibly increase their capability of adsorb contaminants (Frias et al., 2010; Holmes et al., 2014). Holmes et al. (2014) observed a larger amount of adsorbed trace metals in aged pellets of MP, collected in beaches, than in virgin polymers, obtained from a fabric, both for riverwater and seawater mediums. However, beached pellets presented a larger quantity of adsorbed metal when using riverwater as medium. This fact could be an indicative that although salinity does not influence the absorption rate, it is an important parameter when considering the levels of contaminants sorbed onto plastics on different aquatic systems (Holmes et al., 2014).

The potential to carry other contaminants is not the only concern related to MP. The microparticles of plastic can function as substrate to the development of communities of microorganisms, as reported by Zettler et al. (2013), whom characterized microbial communities found attached to plastic marine debris. Furthermore, there is a possibility of the micromaterials carry waterborne pathogens, affecting freshwater quality (Zettler et al., 2013; Wagner et al., 2014; Eerk es-Medrano et al., 2015). Moreover, it was observed that only some marine bacteria have the capability to colonise microplastic particles (Zettler et al., 2013), which can lead to the transport of invasive species to other ecosystems.

Potential impacts on freshwater ecosystems

Biotic impacts

Initial studies demonstrated that freshwater species could ingest MP, confounding them with food, that expose the organisms to contaminants present
in particles surface (Oliveira et al., 2013; Wagner et al., 2014). Nevertheless, each species present a distinct rate of microplastic ingestion, and accumulation, highly related with their habitat, feeding strategy and trophic level (Eerkes-Medrano et al., 2015; Wagner et al., 2014). The microparticles can enter in the organisms in a direct way or indirectly through biomagnification, a process in which micromaterials are transferred along the food chain to higher trophic levels, including birds and mammals (Eerkes-Medrano et al., 2015). Some evidences point to differentiated retention of MP in the organisms associated to their size and density (Brillant & MacDonald, 2000). The accumulation of MP in digestive cavities may affect the total nutrition gain of the organisms since it can cause false satiation or waste of energy reserves in inflammatory responses of tissues to MP, among other negative effects that remain understudied (Oliveira et al., 2013; Wright et al., 2013; Wagner et al., 2014).

Regarding the interaction of MP with vegetable organisms, which is a subject highly uninvestigated, Bhattacharya et al. (2010) demonstrated that in laboratory conditions, freshwater algae Chlorella and freshwater/saltwater Scenedesmus absorb nanoparticles of polystyrene, having a decrease of the photosynthetic activity as consequence. In other hand, Davarpanah & Guilhermino (2015) did not observe an interference on the average specific growth rate of Tetraselmis chuii by polyethelene microspheres.

Abiotic impacts

Apart from interact with organisms, the presence of MP in aquatic systems have consequences that is believed to expand to abiotic environment. Since it is thought that transport potential is quite associated with the concentrations of contaminants in the ecosystems and their residence time, MP are assumed to sorb more contaminants in estuaries, where it has been reported higher concentrations of pollutants, long particle settlement and possible retention in sediments (Bakir et al., 2014; Eerkes-Medrano et al., 2015). In the other hand, MP movements among ecosystems may affect the distribution of chemicals in the environment once they can carry contaminants (Ashton et al., 2010; Bakir et al., 2014; Holmes & Thompson, 2014). Moreover, it was suggested that the accumulation of MP in pelagic and benthic habitats might hinder light penetration into the water column or alter sediments properties, which could have an effect on biogeochemical cycles (Eerkes-Medrano et al., 2015). Nonetheless, these
suggestions are based on preliminary studies. Conducting more detailed work is essential considering that no specific evidences of abiotic effects of MP on marine or freshwater were reported (Eerkes-Medrano et al., 2015).

**Challenges**

MP are classified as emergent pollutants, thus the research related to this issue still needs strong optimization, specially, the ones focus on freshwater environments. This fact hampers an appropriate environmental risk assessment of MP. Actually, freshwater is a threaten natural resource, therefore, the pollution of this type of aquatic systems potentiate their degradation, reducing even more their ecological quality. The biggest challenges include sampling and identification of MP. Charges and the complexity associated to sampling of MP restrict the information collected about their spatial and temporal distributions (Browne et al., 2010; Hidalgo-Ruz et al., 2012; Eerkes-Medrano et al., 2015). Moreover, the identification techniques are time-consuming and do not allow identify all particles (Browne et al., 2010; Galgani et al., 2013; Eerkes-Medrano et al., 2015). The scientific community is aware of this problem and concerned about it, however there still a lot of work to do in order to solve or mitigate it.

The following points are some possible measures that can help to fill the gap of knowledge about the impact of MP in freshwater:

1) Standardize methods of sampling and identification of MP to have a more accurate data and make possible to intercept information of different studies.

2) Apply monitoring studies of MP in freshwater systems, to have a clear idea of the abundance of MP on freshwater.

3) Investigate sources and fate of freshwater MP, to determinate critical points and sinks of MP. In addition, evaluate the potential of rivers to be a source of MP to oceans. For that is important to detect inland sources and understand the degradation processes of plastic debris.

4) Study the dynamic of MP in freshwater systems and their interactions with other contaminants and living organisms. Concentrations of pollutants on marine environments differ significantly from freshwater ones, so the extrapolation of data to freshwater ecosystems is not reliable.
1.2. Phytoremediation

Since the last century, the global economy has based its development on industrial evolution. Therefore, the release of organic and inorganic contaminants, such as petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHS), halogenated hydrocarbons and pesticides, solvents, salt and metals in the environment has been increasing due to anthropogenic activities (Gerhardt et al., 2009; Frias et al., 2010), especially in coastal area as estuaries where the pressure is higher (Almeida et al., 2011; Oliveira et al., 2015). This fact has motivated the scientific community to investigate and develop technologies to remove pollutants from the ecosystems. Phytoremediation is among the suggested methods to recover contaminated regions (Cunningham & Berti, 1993; Pradhan et al., 1998; Alkorta & Garbisu, 2001; Gerhardt et al., 2009; Almeida et al., 2011; Rocha et al., 2014).

Phytoremediation is an ecological technic that resorts to natural functions of the plants to recover soil, sediment, water surface and groundwater environments contaminated with toxic metals, organics and radionuclides (Pradhan et al., 1998). It is defined as “the use of plants and associated rhizospheric microorganisms to remove, contain, or render harmless environmental contaminants” (adapted from Cunningham & Berti, 1993). Phytoremediation is aesthetically, economically and effectively way of remediating contaminated substrates (Cunningham & Berti, 1993; Pradhan et al., 1998; Alkorta & Garbisu, 2001). The benefits of this green technology include low cost, minimal disturbance of the treated area, irrelevant carbon dioxide emissions and removal of a manifold types of contaminants (Pradhan et al., 1998). Further, this treatment method is merely dependent of natural solar energy and plants biological, chemical and physical processes, specifically, absorption, transformation, accumulation, extraction and storage of chemicals present in the environment (Cunningham & Berti, 1993; Pradhan et al., 1998; Alkorta & Garbisu, 2001). In the other hand, there are some limitations associated with this process that needs to be highlighted. The mechanisms involved in the cleanup of the contaminants requires a long period to accomplish satisfactory results, moreover, bioaccumulated contaminants may return to be available in the environment through regular ecological dynamics and finally, the application of new species can affect the biodiversity of the ecosystem (Moosavi & Seghatoleslami, 2013;
Anjum et al., 2014). Beside these restrictions, phytoremediation is an innovative alternative technic that can also be used to attenuate both indoor air pollution and urban smog (Cunningham & Berti, 1993; Moosavi & Seghatoleslami, 2013).

**Categories of phytoremediation**

Phytoremediation is a technology whose application can follow different strategies (Vishnoi & Srivastava, 2008; Anjum et al., 2014; Ali et al., 2013; Moosavi & Seghatoleslami, 2013). Specifically, phytoextraction is a process that involves the uptake of contaminants from the affected area via plant roots and their translocation to aboveground plant tissue (Anjum et al., 2014). In turn, phytostabilization is the application of plants in contaminated regions to control the concentration of contaminants in the medium. It limits the mobility and bioavailability of pollutants in the environment since they remain attached to plants roots or confined in the soil around plant roots (Moosavi & Seghatoleslami, 2013). Phytovolatilization is a process based on the capacity of the plants to convert captured pollutants from soil or water to a volatile form, releasing them to the atmosphere. Nevertheless, the efficacy of phytovolatilization is quite questionable considering that it only transfers pollutants from a substrate to the atmosphere (Ali et al., 2013; Anjum et al., 2014). The metabolic process that allows plants to detoxify organic xenobiotics in their tissues is referred as phytodegradation, it is an enzymatic degradation not dependent on microorganisms associated with the rhizosphere (Ali et al., 2013; Vishnoi & Srivastava, 2008). Beyond that, some metabolic activities of the plants may enhance the biodegradation of organic pollutants by microorganisms present in the soil, the denominated rhizodegradation or rhizoremediation (Vishnoi & Srivastava, 2008; Ali et al., 2013; Ribeiro et al., 2014). This process occur when plants release through the roots nutritive compounds, such as sugars, amino acids, growth factors, which stimulate the development of large and diverse microbial populations. That may lead to an increase of contaminants biodegradation in the soil surrounding roots (Vishnoi & Srivastava, 2008).

**Phytoremediation of organic contaminants**

There are evidences that phytoremediation coupled with rhizodegradation is an effective way of remove and degrade organic contaminants from affected areas (Alkorta & Garbisu, 2001; Vishnoi & Srivastava, 2008; Gerhardt et al., 2009;
Ribeiro et al., 2014). The base for application of this method is the verification that metabolization of organic chemicals occurs faster in vegetated soils compared with non-vegetated soils (Pradhan et al., 1998; Alkorta and Garbisu, 2001). For instance, Ribeiro et al. (2014) observed a higher hydrocarbon degrading microbial abundance in sediments colonized with salt marsh plants than in un-colonized sediments. They also noted that, beyond the connexions between plants and microorganisms, plant characteristics and environmental conditions influence the remediation process, insofar as they can limit root growth, which in turn influence availability and distribution of nutrients in the system (Ribeiro et al., 2014). Furthermore, organic contaminants, like polychlorinated biphenyls (PCBs) and PHAs, have a structure similar to natural aromatic plants compounds which allows the processes of bioremediation (Gerhardt et al., 2009).

Phytoremediation of inorganic contaminants - metals

Metals, contrarily from organic substances, are non-biodegradable, being persistent in the environment. The ecological contamination by metals has been increasing along the years, which can have negative impacts on environmental and human health (Ali et al., 2013; Moosavi & Seghatoleslami, 2013). Studies on application of phytoextraction to remove metal and metalloids from soils or sediments have been demonstrated satisfactory results (Silva et al., 2014). Nevertheless, some factor may limit the efficacy of this technic, specifically bioavailability of the metal in soil, soil properties, metal speciation and plants characteristics, being essential plants tissues be tolerant to metals (Ali et al., 2013). Phytoremediation of metals involves translocation of metals to plants roots and storage in biomass. Metals are normally accumulated in the roots or mobilized to the leaves (Ali et al., 2013; Silva et al., 2014).

Phytoremediation of copper and cadmium by the salt marsh plant Phragmites australis

Phragmites australis is one of the salt marsh plants with potential to phytoremediate metal contaminants in estuarine sediments. In fact, several salt marsh plants have shown capacity to accumulate metals from contaminated sediments contributing for their recovering (Al-Taisan, 2009; Almeida et al., 2011; Rocha et al., 2014; Silva et al., 2014).
*P. australis* is a common reed of Poaceae family, globally spread in wetlands of temperate and tropical areas. It is an invasive species with capacity to support a manifold of different wet habitats conditions, except regions with extreme conditions of nutrient deficiency or high salinity (Havens *et al.*, 1997; Al-Taisan, 2009).

Several studies verified that *P. australis* has the capacity of accumulate copper (Cu) and cadmium (Cd) in its belowground and aboveground tissues (e.g. Al-Taisan, 2009; Almeida *et al.*, 2011; Oliveira et al, 2013; Silva *et al.*, 2014).

It is know that Cd, contrary from Cu, is not an essential element for *P. australis*. Since chemical properties of Cd are similar to zinc (Zn), is possible that Cd enter to root cell through mechanisms used by plants to uptake Zn, a micronutrient (Silva *et al.*, 2014).

### 1.3. Objectives

The aim of this work was to investigate possible interferences of MP in phytoremediation of sediments contaminated with trace metals, Cu and Cd, by the salt marsh plant *P. australis*. Another complementary objective was to verify if physical properties (type and aging) and concentration of MP affect their capacity to sorb metals.

To achieve the proposed objectives, an experimental assay with sediment elutriates was carried out. Elutriate is a simple medium that simplistic mimics estuarine conditions, simulating natural interactions between sediments, estuarine water and plants. For that, salt marsh plants (*Phragmites australis*) were exposed to elutriate solution doped with a metal (Cu or Cd) or with a metal plus microplastics of polyethylene (PE) in the presence or in the absence of sediments. PE were used as a MP model.

To investigate the interactions between MP and metals, preliminary experiments only with elutriate solutions were performed. These experiments had the objective of testing the influence of: i) different types of MP (PE, fishing line fibers, MP of film and MP of bottle cap); virgin MP and aged MP; (iii) salinity; and (iv) concentration of MP in solution on adsorption of Cu and Cd.
Chapter II

2. Methods

2.1. Adsorption of metals by MP

2.1.1. Adsorption of metals by polyethylene microspheres (PE) – influence of PE concentrations

2.1.2. Adsorption of metals by different types of MP

2.1.3. Adsorption of metals by different types of aged MP

2.1.4. Impact of salinity on absorption/adsorption of metals by MP

2.1.5. Metal analysis in solution

2.2. Impact of MP in phytoremediation of metals by estuarine plants

2.2.1. Sampling

2.2.2. Elutriate

2.2.3. Microcosm experiments

2.2.4. Experimental assays

2.2.5. Analytical determinations

2.2.5.1. Material and reagents

2.2.5.2. Metal levels

2.3. Statistical analysis
2. Methods

The incubation time (7 days) for all experiments was established to simulate an acute contamination scenario. Besides, this timeframe was also selected accordingly to previous studies, as longer time periods would result in plant damages, which can biased experimental results (Rocha et al., 2014). The medium used was elutriate solution, which been used in previous studies showing to be suitable to investigated plant-estuarine water-sediment interactions (Rocha et al., 2014; Almeida et al., 2008) (section 2.2.).

2.1. Adsorption of metals by MP

To investigate the interactions of MP with Cu and Cd, different laboratory experiments were performed. All treatments were done in triplicate.

2.1.1. Adsorption of metals by polyethylene microspheres (PE) – influence of PE concentrations

PE – Pink Microsphere, Polyethylene (Fig. 1) (d=1.00g/cc, from Cospheric Innovations in Microtechnology) were suspended in an aqueous medium doped with Cu or Cd (10 mg/L or 1 mg/L respectively) during 7 days. Solution was sampled at days 1, 3 and 7 to follow the progress of dissolved metal levels in medium during the experiment (please see following section 2.1.5 for further details). To simulate natural interactions that occur between sediments, estuarine water and MP, elutriate solutions were used as aqueous medium. Three different groups with distinct amounts of PE were used to verify if the quantity of MP influenced the Cu or Cd adsorption by MP. PE microspheres were used as a MP model as this is a very commonly used plastic type, used already in this form.

In total, for each metal, 12 experimental vessels were used: 3 flasks with elutriate and metal, composed the control group; 3 flasks with elutriate, metal and the lowest concentration of PE – 150 mg/L; 3 flasks with elutriate, metal and PE -1500 mg/L; 3 flasks with elutriate, metal and the highest concentration of PE – 15000 mg/L.
2.1.2. Adsorption of metals by different types of MP

To investigate if the properties of MP interfere with metal adsorption by MP, different types of MP (all in a concentration of 1500 mg/L) were exposed to an elutriate solution doped with Cu (10 mg/L) or Cd (1 mg/L) (Fig. 2).

The 4 types of MP used correspond to those that are commonly found in the environment: virgin microsphere of polyethylene (PE), fishing line fibers (fibers), MP of film plastic bags (film), MP of bottle cap (Eerkes-Medrano et al. 2015; Wagner et al., 2014). Apart of PE, all the MP were obtained by breaking large items of plastics through manual procedures and washed with deionised water and emerged in 20% (v/v) HNO₃ solution during approximately 1 hour to remove possible attached metals.

A total of 15 experimental vessels were prepared: 3 control flasks with elutriate and metal; 3 flasks with elutriate, metal and PE; 3 flasks with elutriate, metal and fibers; 3 flasks with elutriate, metal and film; 3 flask with elutriate, metal and MP of bottles cap.

Experiments ran for 7 days and medium samples were collected at days 1, 3 and 7 for further metal concentration analytical determination (please see following section 2.1.5 for further details).
2.1.3. Adsorption of metals by different types of aged MP

The experiment described above in section 2.1.2. was repeated using aged MP (Fig. 3). The aging process was achieved by setting each type of MP (1500 mg/L) in a beaker with 400 mL of river water. Those beakers were agitated by an orbital shaker for 2 weeks at a constant velocity of 50 units per minute. After the aging time, MPs were washed with deionised water and emerged in 20% (v/v) HNO₃ solution during approximately 1 hour (except fibers that stayed emerged in the acid during 30 minutes). Hereafter, MPs were removed from the beakers, washed with deionised water and left immersed in deionized water for a day. Then, they were dried in a lab oven at 30 ºC, for 5 days.
2.1.4. Impact of salinity on adsorption of metals by MP

PE, in a concentration of 1500 mg/L, were suspended in deionized water, doped with Cu (10 mg/L) or Cd (1 mg/L), containing different concentrations of NaCl, to investigate if salinity levels can influence the absorption/adsorption of metals by MP. The following NaCl concentrations (g/L) 0, 5, 10, 15, 20, 25, 30 and 35 were used, and the experiment lasted 7 days. Similarly to previous experiments, samples were taken at days 1, 3 and 7 to assess the evolution of dissolved metal concentrations in the medium throughout the experiment. Each concentration corresponded to an experimental unit. To create replicates, in all sampling days, three samples were collected from each flask.

2.1.5. Metal analysis in solution

In experiments with Cu, 1 mL of elutriate solution or deionized water with different concentrations of NaCl were collected from each flask and immediately acidified with HNO₃ (1 % of the total amount of solution collected) and diluted ten times. In assays with Cd, 2 mL of elutriate solution or deionized water with different concentrations of NaCl were collected from each vessel, being also acidified with HNO₃, in a concentration of 1 % of the total amount of solution collected. All solutions were kept refrigerated at 4 ºC until analysis. Afterwards, metal levels 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. Impact of MP in phytoremediation of metals by estuarine plants

2.2.1. Sampling

The sampling of P. autralis and sediment was carried out in October 13th 2015 for the assay with Cu and in May 2016 for the assay with Cd from the salt marsh area (Fig. 4) of the Lima River estuary (Viana do Castelo, Portugal), at low tide. This estuary is located in an urban and industrialized region, and
consequently exposed to several stress factors, such as fishing, agricultural run-off, navigation activity in the lower estuary, cellulose factory in the upper estuary and respective sewage discharges (Almeida et al., 2011; Ramos et al., 2015).

Only green plants *P. australis* without a senescent appearance and with similar size and age were collected in cubes of approximately 10 cm x 10 cm x 10 cm to preserve the plant rhizosphere environment. Immediately after, sediments of rhizosphere environment were separated from the plant roots and stored in plastic bags. Individual plants (compounded by leaves, stems, rhizomes and roots) were rinsed with estuarine water and stored in plastic bags. Finally, estuarine water was collected in plastic bottles.

2.2.2. Microcosm experiments

For both Cu and Cd, the assay with *P. australis* followed an experimental design similar to the one described in Almeida et al. (2008) and lasted seven days. Two different systems were used (Fig. 5). One system had plants and sediment soaked in its respective elutriate solution, which simulates estuarine conditions, since it allows exchange of nutrients and contaminants between plants, solution and sediment. The other system was simpler, including only
elutriate solution and plants. In this late system, plants’ roots were only in contact with elutriate, therefore, dynamics of nutrients and contaminants was limited to plant roots and solution (Almeida et al., 2008).

2.2.3. Elutriate

Elutriate solutions were obtained following USEPA protocol (1991). Several flasks, each containing 200 mL of estuarine water and 50 g of wet sediment, were shaken during 30 minutes in an agitator at velocity of 100 units per minute. Afterwards, flasks were placed to rest for 12 h. To finish, half of the mixture was decanted, to separate elutriate from sediment, and filtered by 0.45 µm pore size filters (cellulose nitrate membrane, Millipore), removing particulate matter (except colloids) and decreasing the concentration of microorganisms present in solution. Another half was ready to use after the resting time.

These types of solutions simulate interactions between water and sediments, giving an idea of the quantity of metal that is transferred from one medium to another. In estuarine areas, these exchanges can occur during increase of river flow, like floods, strong tides, regional sea level rise or dredging operations (Almeida et al., 2008). Moreover, elutriate is a simple medium that enables an easier data interpretation. Mediums composed by sediments soaked with elutriate simulates estuary conditions, since it provide circumstances to verify nutrients and contaminants exchange between plants, solution and sediment.

2.2.4. Experimental assays

For each metal, 6 flasks containing 3 individual plants (P. australis) each and elutriate solution doped with metal were prepared, in the following concentrations: Cu – 10 mg/L or Cd – 1 mg/L (Fig 5). These levels of metal were selected based on previous studies (Rocha et al., 2014) which showed that the used concentrations do not cause any visual adverse effect, despite they are higher than their respective effect range – median (ERM) (270 µg/g_DW for Cu and 9
µg/g DW). ERM is the sediment quality guideline that indicates the pollutant concentration above which adverse biological effects may frequently occur in marine and estuarine sediments (Long et al., 1995). Although these concentrations are unlikely to be found in the environment, they may cause a more pronounced effect on plants, producing measurable results in short term experiments (Rocha et al., 2014). To 3 of those flasks, PE were added, in a concentration of 1500 mg/L. Another 6 flasks were prepared, each also with 3 individual plants, containing sediment soaked in its elutriate also doped with Cu or Cd. Each flask was wrapped with aluminium foil to protect plants’ roots from sunlight and simulate natural conditions of rhizosphere environment. The flasks remained in a protected area of the laboratory, with a glass roof to allow the penetration of sunlight and the natural photoperiod regime, for a period of one week. Afterwards, plants were removed from the flasks, washed with deionized water and divided in four parts: roots, rhizomes, stems and leaves. Elutriates were decanted and sediments were collected. All plant tissues and sediments were dried at room temperature until constant weight. Solutions were acidified and refrigerated at 4 ºC until analysis.

Figure 5 Experimental design of the assay with plants
2.2.5. Analytical determinations

2.2.5.1. Material and reagents

To prevent contamination, all sampling and labware materials were soaked in 20% (v/v) HNO$_3$ solution for at least 24h, rinsed several times with bi-deionised water (conductivity < 0.1 µS cm$^{-1}$) and dried in an oven at 30 ºC. Suprapure concentrated HNO$_3$, from Merck, was used without further purification.

2.2.5.2. Metal levels

To measure metal levels in sediments, to ca. 0.25 g of dry sediment 5 mL of concentrated HNO$_3$ were added. To measure the metal levels in plant tissues, to ca. 0.50 g of dry plant sample 5 mL of a 30 % H$_2$O$_2$ solution and 1 mL of concentrated HNO$_3$ were added. Then, samples were digested in a high pressure microwave system (Ethos, Milestone) with Teflon vessels with a previously optimized temperature programme (Almeida et al., 2004). For elutriate solutions, to 10 mL of each sample 1 mL of concentrated HNO$_3$ were added.

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

2.3. Statistical analysis

Cu and Cd mean values and respective standard deviation (n=3) were calculated for each treatment. Data were analysed statistically using 1-way Analysis of Variance (ANOVA) and Tukey pair wise comparisons test were applied to determine the significance of differences among treatments. Unpaired t-student test (p<0.05) was employed to investigate significant differences between control and one selected treatment. The statistical program used was Dell Statistica 13 software and the confidence limit was 95%.
Chapter III

3. Results

3.1. Adsorption of Cu by Microplastics
   3.1.1. Influence of PE concentration on Cu adsorption
   3.1.2. Adsorption of Cu by different types of microplastics
   3.1.3. Adsorption of Cu by different types of aged microplastics
   3.1.4. Impact of salinity on adsorption of Cu by microplastics

3.2. Impact of microplastics in phytoremediation of Cu by the estuarine plant *P. australis*
   3.2.1. Cu levels in the tissues of plants
   3.2.2. Concentration of Cu retained in the medium

3.3. Adsorption of Cd by Microplastics
   3.3.1. Influence of PE concentration on Cd adsorption
   3.3.2. Adsorption of Cd by different types of microplastics
   3.3.3. Adsorption of Cd by different types of aged microplastics
   3.3.4. Impact of salinity on adsorption of Cd by microplastics

3.4. Impact of microplastics in phytoremediation of Cd by the estuarine plant *P. australis*
   3.4.1. Cd levels in the tissues of plants
   3.4.2. Concentration of Cd retained in the medium
3. Results

3.1. Adsorption of Cu by MP

3.1.1. Influence of PE concentration on Cu adsorption

The levels of Cu in elutriate solution (Fig. 6) of all treatment groups (PE concentrations: 150 mg/L, 1500 mg/L and 15000 mg/L), including the control (without PE), varied along the experiment period, with a general tendency to decrease over time. Specifically, in the control group, there was a significant (ANOVA control: F=28.3 p<0.05) decrease of Cu from day 1 to day 3. However, at the end of the experiment (day 7), Cu concentrations in elutriate solutions with MP were not significantly (ANOVA 150 mg PE/L: F=3.6 p>0.05; ANOVA 1500 mg PE/L: F=3.8 p>0.05) lower than in the day 1, with the exception of treatment 15000 mg PE/L, for which Cu concentration decreased significantly (ANOVA: F=25.9 p<0.05) from day 1 to day 7.

Regarding the influence of PE concentration on Cu adsorption, comparing with the control group, at day 1 only the highest PE concentration (15000 mg/L) presented significantly higher Cu concentration in solution (ANOVA day 1: F=4.4 p<0.05). At day 3, Cu levels were significantly (ANOVA day 3: F=37.8 p<0.05) different among all groups, with Cu levels in solution increasing with increasing amount of PE. At day 7, Cu concentration in the control group was significantly (ANOVA day 7: F=34.5 p<0.05) lower than for all PE concentrations. So, in general, after 7 days, Cu concentration in solution tended to be higher in the presence of PE, independently of PE amounts.
3.1.2. Adsorption of Cu by different types of MP

The Cu levels in elutriate solutions doped with Cu (10 mg/L) in absence (control) and presence of different types of MP (PE, bottle cap, fibbers and film) showed once again a decreasing trend along time (Fig. 7). However, this trend was significant only for fibers and film MP, which showed significantly (ANOVA fibers: $F=19.4$ $p<0.05$; ANOVA film: $F=20.1$ $p<0.05$) higher Cu concentration at day 1 comparing to other days of the experiment.

At day 1, Cu levels of elutriate solution with fibers, and film were significantly lower (ANOVA day 1: $F=16.7$ $p<0.05$) than Cu levels in the control. For day 3 and day 7, only the film MP registered a significantly lower Cu concentration in solution than control (day 3 ANOVA: $F= 3.1$ $p>0.05$; day 7 ANOVA: $F=8.2$ $p<0.05$).
3.1.3. Adsorption of Cu by different types of aged MP

The concentrations of Cu on elutriate solution without (control group) and with aged MP (aPE, abottle cap, afibers and afilm) decreased along time (Fig.8). Specifically, levels of Cu in elutriate with afibers were significantly (ANOVA aFibers: F=16.4 p<0.05) higher in day 1 comparing to other days, however without significant (ANOVA aFibers: =16.4 p>0.05) differences between days 3 and 7. At the end of the experiment (day 7), the elutriate solution with aPE presented significantly lower Cu concentrations than in the day 1 (ANOVA aPE: F=32.6 p<0.05). Cu concentrations in elutriate solution containing afilm decreased significantly along all days of the experiment (ANOVA afilm: F=123.3 p<0.05).

At day 1, Cu concentration of elutriate with afilm was the only one significantly lower than control treatment (ANOVA day 1: F=6.2 p<0.05) and no significant differences were observed for the other types of aged MP (Fig. 3).
day 3, all types of aged MP except aPE registered significantly lower Cu concentrations than control (ANOVA day 3: F=33.5 p<0.05). In the last day (day 7), only the elutriate solution with afilm presented significantly lower Cu levels than control (p<0.05).

3.1.4. Impact of salinity on adsorption of Cu by MP

The Cu levels in deionised water amended with Cu, PE and different concentrations of NaCl are shown in Fig. 9. In general, there were no temporal variations of Cu concentration within each salinity group. The exception was at day 7 for which Cu concentrations were significantly lower at salinity 25 (ANOVA: F=14.8 p<0.05) and 30 (ANOVA: F=12.3 p<0.05) NaCl g/L, comparing with the first day of the experiment.

In general, there were also no significant different among different salinities in each day. At day 1, the concentration of Cu in solution with 10 g/L of NaCl was significantly (ANOVA day 1: F=5.1 p<0.05) lower than in the solution

![Figure 8](image-url) Concentrations of Cu (mean and standard deviation, n=3) in elutriate solutions doped with Cu (10 mg/L) in absence (control) and presence of different types of aged MP (all in a concentration of 1500 mg/L) after 1, 3 and 7 days of experiment. Different letters indicate significant differences between different types of MP for the same day (p<0.05).
without salinity (0 g/L). At day 3, only for salinity 5 g/L a significant (ANOVA day 3: F=10.96 p<0.05) difference on Cu levels relatively to solution without salinity was observed. But at day 7, no significant (ANOVA day 7: F=0.52 p>0.05) differences among different salinities were observed.

3.2. Impact of MP in phytoremediation of Cu by the estuarine plant *P. australis*

*P. australis* was exposed to elutriate solution doped with Cu, with or without MP, with or without sediments for 7 days. Afterwards, levels of Cu were measured in different matrices, as tissues of the plant, sediments and elutriate solutions.

3.2.1. Cu levels in the tissues of plants

Different plant tissues presented distinct levels of Cu (Fig. 10). Plants accumulated a higher amount of Cu in belowground tissues (roots and rhizomes) than in aerial tissues (stems and leaves). In fact, Cu concentrations in roots ad
rhizomes were, respectively ca. 200 and 60 times higher than Cu concentration in stems and leaves in the absence of sediments and ca. 30 and 6 times higher, respectively, in the presence of sediments. Comparing with the roots, rhizomes accumulated lower amount of Cu.

Relatively to control group (which represent the Cu level initially present in plants) in general significantly (ANOVA roots: F=16.6 p<0.05; ANOVA rhizomes: F=8.7 p<0.05) higher amounts of Cu were also observed in belowground tissues of exposed plants (Fig. 10A). The levels of Cu detected in aerial tissues were in general not significantly (ANOVA stems: F=0.5 p>0.05; ANOVA leaves: F=0.7 p>0.05) different from the ones in control group (Fig. 10B).

Particularly, the values of Cu detected in plants exposed to elutriate solutions without sediments (i.e. treatments Cu and Cu+MP) were significantly (ANOVA Cu: F=30.9 p<0.05; ANOVA Cu+MP: F=22.3 p<0.05) higher in the roots. Moreover, there was not observed significant (ANOVA roots: F=16.6 p>0.05; ANOVA rhizomes: F=8.7 p>0.05; ANOVA stems: F=0.5 p>0.05; ANOVA leaves: F=0.7 p>0.05) differences in levels of Cu in the presence of MP. Comparing with the roots, rhizomes accumulated lower amount of Cu. However, the amounts of Cu stored in rhizomes were still higher than the concentrations detected in stems and leaves, without influence of the presence of MP.

In the presence of sediments, the belowground parts accumulated a lower quantity of Cu, which was independent of the presence of PE. Nevertheless, Cu concentrations detected in those tissues were also higher than the ones found in stems and leaves. Roots' tissues presented a significant (ANOVA roots: F=16.6 p<0.05) higher concentration of metal in both treatments (Cu+Sed and Cu+PE+Sed). In turn, rhizomes accumulated significantly (ANOVA rhizomes: F=8.7 p<0.05) higher Cu levels in the absence of PE, comparing with control group. Concentration values detected on rhizomes were lower than the ones observed on roots, but higher than the ones observed on aerial tissues.
3.2.2. Concentration of Cu retained in the medium

The levels of Cu in elutriate solutions and sediments amended with Cu or Cu+PE are presented in Fig. 11.

Without sediments, Cu content in solution (Fig. 11A) was significantly (ANOVA elutriate: F=10.7 p<0.05) higher in the presence of MP. In the presence
of sediment, the same did not occur in the elutriate solution, where no significant (ANOVA elutriate: F= 10.7 p>0.05) differences were observed.

For sediments (Fig. 11B), no significant (t-test sediments: T= -2.6 p>0.05) differences between the treatments were observed.

**Figure 11** Cu concentrations (mean and standard deviation, n=3) in elutriate solutions (A) with or without sediments (sed.) and in sediments (B) amended with Cu (10 mg/L) or Cu+PE (10 mg/L and 1500mg/L, respectively; PE – polyethylene microspheres), after a 7 days assay with *P. australis*. Different letters indicate significant differences among treatments (p<0.05).
3.3. Adsorption of Cd by MP

The effect of MP concentration, type and aging, as well as the effect of salinity on the possible removal of the metal from solution due to adsorption to MP were investigated by different experimental assays of 7 days of exposure. With the exception of salinity tests (carried out with deionized water), experiments were carried out with elutriate solution. During the experiments, levels of Cd in solution were measured on days 1, 3 and 7.

3.3.1. Influence of PE concentrations on Cd adsorption

The levels of Cd in elutriate solutions (Fig 12) of all treatment groups (PE concentrations: 150 mg/L, 1500 mg/L and 15000 mg/L), including the control (without PE), did not vary along the experimental period (ANOVA day1: F=0.23 p>0.05; ANOVA day3: F=1.3 p>0.05; ANOVA day7: F=0.37 p>0.05).

In general, there were no significant variations throughout the days for each treatment (ANOVA control: F=3.2 p>0.05; ANOVA 150 mg PE/L: F=6.2 p<0.05; ANOVA 1500 mg/L: F=1.6 p>0.05; ANOVA 15000 mg/L: F= 1.7 p>0.05).

![Figure 12](image_url)

Figure 12: Concentrations of Cd (mean and standard deviation, n=3) in elutriate solutions doped with Cd (1 mg/L) in absence (control) and presence of different concentration of polyethylene microspheres (PE); sampled at days 1, 3 and 7 of experiment.
3.3.2. Adsorption of Cd by different types of MP

The Cd levels in elutriate solutions doped with Cd in absence (control) and presence of different types of MP (PE, bottle cap, fibbers and film) showed an increasing trend along time (Fig. 13). Cd concentration of control group increased significantly along all the days of the experiment (ANOVA control: F=102.2 p<0.05). The levels of Cd in elutriate solution with MP of bottle cap were significantly higher (ANOVA bottle cap: F=12.33 p<0.05) in the last day of the experiment. Moreover, Cd concentration on elutriate with film increased significantly (ANOVA film: F=29.1 p<0.05) at day 3 but stabilized in day 7.

At day 1, Cd levels of elutriate solution with PE, were significantly higher (ANOVA day 1: F=5.5 p<0.05) than the control Cd levels. Up to the end of the experiment, only the solution with film MP registered a significantly lower Cd concentration comparing with the control solution (ANOVA day 3: F= 10.2 p<0.05; ANOVA day 7: F=15.1 p<0.05).

Figure 13 Concentrations of Cd (mean and standard deviation, n=3) in elutriate solutions doped with Cd (1 mg/L) in absence (control) and presence of different types of MP (all in a concentration of 1500 mg/L), after 1, 3 and 7 days of experiment. Different letters indicate significant differences among treatments (p<0.05).
3.3.3. Adsorption of Cd by different types of aged MP

In general, the concentrations of Cd on elutriate solution without (control group) and with different types of aged MP (aPE, abottle cap, afibers and afilm) (Fig. 14) did not varied significantly between treatment groups, nor along the experimental time (ANOVA day1: F=0.7 p>0.05; ANOVA day3: F= 0.1 p>0.05; ANOVA day 7: F=3.6 p<0.05).

Figure 14 Concentrations of Cd (mean and standard deviation, n=3) in elutriate solutions doped with Cd (1 mg/L) in absence (control) and presence of different types of aged MP (all in a concentration of 1500 mg/L), after 1, 3 and 7 days of experiment.

3.3.4. Impact of salinity on adsorption of Cd by MP

In general, Cd concentration within each salinity group decreased among the days (Fig. 15). At day 7, with exception of solution with 20 g/L that presented a significant decrease from day 1 to day 3 (ANOVA 20 g/L: F= 6.74 p<0.05) but stabilized at day 7, all treatment groups, including control, presented significant lower levels of Cd (ANOVA 0 g/L: F=17.9 p<0.05; ANOVA 5 g/L: F=59.6 p<0.05;
ANOVA 10 g/L: 13.1 p<0.05; ANOVA 15 g/L: F=40.7 p<0.05; ANOVA 25 g/L: F=81.5 p<0.05; ANOVA 30 g/L: F=20.4 p<0.05; ANOVA 35: F=185.9 p<0.05) relatively to day 1.

Small changes occurred among different salinity at each day. At day 3, Cd levels on solution with 10 g/L were significantly higher than the ones on the control (ANOVA day 3: F=14.2 p<0.05). At day 7, concentrations of Cd in solutions with 10, 15 and 20 NaCl g/L were significantly higher than the one in control solution (ANOVA day 7: F=14.2 p<0.05).

Figure 15 Concentrations of Cd (mean and standard deviation, n=3) in deionized water amended with different concentrations of NaCl and PE (1500 mg/L), after 1, 3 and 7 days of experiment. Different letters indicate significant differences among treatments (p<0.05) at same day.

3.4. Impact of MP in phytoremediation of Cd by the estuarine plant *P. australis*

*P. australis* was exposed to elutriate solution doped with Cd (1 mg/L), with or without PE, with or without sediments, for 7 days. Afterwards, levels of Cd were measured in plants' tissue, in sediments, when present, and in elutriate solutions.
3.4.1. Cd levels in plants’ tissues

Levels of Cd were only detected on belowground tissues, roots and rhizomes, of plants exposed to doped elutriate solutions, with or without sediments (Fig. 16). Plants of the control group (plants not exposed to elutriates, which represent the concentration of Cd initially present in plants) presented Cd concentrations very close to the limit of detection (Table 1).

In general, significantly (t-test Cd: t-value = 4.7 p<0.05; t-test Cd+MP: t-value = 7.2 p<0.05; t-test Cd+Sed: t-value = 10.3 p<0.05; t-test Cd+MP+Sed: t-value = 2.8 p<0.05) higher amounts of Cd were accumulated in the roots, in comparison with rhizomes. However, there were no significant (ANOVA roots: F=12.3 p>0.05) differences in Cd levels in the roots of exposed plants in the presence or absence of PE, either with or without sediments. A similar scenario was observed for Cd concentrations in rhizomes (ANOVA rhizomes: F=1.4 p>0.05).

![Figure 16 Cd concentrations (mean and standard deviation, n=3) in tissues of plants (roots and rhizomes) after 7 days of exposure to elutriate solutions not amended, control, and amended with Cd (1 mg/L) or Cd+PE (1 mg/L and 1500mg/L, respectively; PE = polyethylene microspheres), in presence or absence of sediments (Sed).](image)

Regarding plants aboveground tissues, there was a slightly increase of Cd in the stems of plants exposed to elutriate doped with Cd in the absence of sediments (Table 1). However, once again there were no significant differences
between Cd concentrations in the presence or absence of PE MPs. For leaves, there was no Cd accumulation.

**Table 1** Cd concentrations (mean and standard deviation (in parenthesis, n=3)) in plants’ aboveground tissues (stems and leaves) after 7 days of exposure to elutriate solutions amended with Cd (1 mg/L) or Cd+PE (1 mg/L and 1500mg/L, respectively; PE – polyethylene microspheres), in presence or absence of sediments (Sed). Limit of detection (LD) = 0.7 µg/g).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control</th>
<th>Cd</th>
<th>Cd+PE</th>
<th>Cd+Sed</th>
<th>Cd+PE+Sed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stems</td>
<td>1.4 (0.3)</td>
<td>2.3 (0.1)</td>
<td>2.1 (0.5)</td>
<td>2 (1)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.2 (0.2)</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
</tbody>
</table>

### 3.4.2. Concentration of Cd in the medium

The levels of Cd in elutriate solutions were below of limit of detection (LD=0.042 mg/L). Thus it was only possible to measure Cd levels (ca. 5 µg/g) on sediments amended with Cu or Cu+PE (Fig. 17) and no significant (t-test Sed: t-value = 0.5 p>0.05) differences between the treatments were observed.

![Figure 17](image-url) **Figure 17** Cd concentrations (mean and standard deviation, n=3) in sediments amended with Cd (1 mg/L) or Cu+PE (10 mg/L and 1500mg/L, respectively; PE – polyethylene microspheres), after a 7 days assay with *P. australis*.
Chapter IV

4. Discussion

4.1. MP Impact of MP in phytoremediation of Cu by *P. australis*

4.2. Impact of MP in phytoremediation of Cd by *P. australis*
4. Discussion

Estuaries are highly productive ecosystems (Costanza et al. 1997), and typically host salt marshes settled on intertidal areas of mud and sand flats which are stabilized by vegetation and periodically submerged by daily tidal flow (McLusky & Elliott, 2004; Almeida et al., 2013). Estuaries constitute a transitory environment from river to sea, where is possible to observe changes on surrounding areas along the way. The conservation of estuaries and salt marshes is important to not only maintain and conserve biodiversity, but also to stabilize coastal environments and preserving estuarine functionality, which is highly dependent on salt marshes (Almeida et al., 2013). However, estuaries have been exploited by humans in an unsustainable way (McLusky & Elliott, 2004), and intensive agriculture and industrial activities are some of the examples of factors impacting estuarine ecological health (Ramos et al., 2015). Moreover, salt marshes act as sink to several types of pollutants, as metals, which are retained in their sediments (Almeida et al., 2011; Vianello et al., 2013). Moreover, MP can also be retained in sediments of these areas (Eerkes-Medrano et al., 2015; Wagner et al., 2014).

MP constitute a major problem in plastic waste issue, since they are not only widely spread in the environment, but also more difficult to monitor, and not least, they have an enormous impact at a chemical and physical level on ecosystems and human health (Duis & Coors, 2016; Eerkes-Medrano et al., 2015; Wagner et al., 2014). All these problems are due to MP small size and large volume-surface area ratio (EU Commission, 2011). Furthermore, the research made about these pollutants is still scarce, especially in what concerns the impact of MP on freshwater environments, and on ecosystem’s functions, such as phytoremediation of metals by salt marshes plants. Several studies reported *P. australis* capability of phytoremediate Cu and Cd polluted sediments (e.g. Almeida et al., 2004, 2011 and 2013, Silva et al., 2014), however the interactions between metals with MP and how the presence of MP influence metal removal by plants from soil still needs to be investigated.
4.1. Impact of MP in phytoremediation of Cu by \textit{P. australis}

Before the assay with plants, preliminary experiments were carried out to study the interactions of MP with the metal, assessing possible adsorption of Cu to polyethylene microspheres (PE). PE was used as model for the study due to its importance as one of the most produced type of polymer. In fact, they are commonly used in the industry and, consequently, are among the most spread type of MP in the environment (EU Commission, 2011; Frias \textit{et al.}, 2010; Wagner \textit{et al.}, 2014). PE are widely documented as a component of marine and freshwater plastic debris (Eerkes-Medrano \textit{et al.}, 2015; EU Commission, 2011; Frias \textit{et al.}, 2010; Harrison \textit{et al.}, 2014; Wagner \textit{et al.}, 2014). In these experiments elutriate solutions were used as medium since they simulate, in a simple way, the interactions between water, sediments and pollutants in an estuarine environment (Almeida \textit{et al.}, 2008).

First, different concentrations of PE were added to elutriate solutions doped with Cu, during 7 days to investigate the influence of MP concentration on the adsorption of Cu by PE. The PE concentrations used were selected based on several parameters, including the levels of PE found in estuarine areas (Eerkes-Medrano \textit{et al.}, 2015). Also, the concentration of 1500 mg PE/L is within the interval of concentrations normally used in laboratory experiments with MP (e.g. Bakir \textit{et al.}, 2014; Brennecke et al, 2016; Holmes \textit{et al.}, 2014; Nobre \textit{et al.}, 2015). The concentrations 150 mg/L and 15000 mg/L are, respectively, one order of magnitude lower and one order of magnitude higher than 1500 mg/L. Results showed no adsorption of Cu by PE. In fact, Cu levels in solution were higher in the presence of MP than in the absence of MP. These results are different from Holmes \textit{et al.} (2012), which showed that PE pellets were capable of adsorbing Cu in river or seawater. Elutriate solution matrix can be more complex, i.e. including more components than river or sea water matrix, having for instance, considerable amounts of colloidal species such as humic and fulvic acids, which can affect metal interactions. In fact, the decreasing trend in Cu concentration over time, observed particularly in control group, can be explained by the occurrence of aggregation and adsorption of Cu by colloid particles in suspension (Almeida \textit{et al.}, 2008; Im & Jung, 2015), which resulted in a lower detection of soluble Cu (only soluble Cu was measured). In the presence of MP, the amount of soluble Cu increased in solution relatively to control (absence of MP) probably because MP and colloids adsorbed each other,
preventing the formation of agglomerates of Cu and colloids. The use of more or less amount of PE in solution had similar results, with none of the conditions indicating adsorption of Cu by PE. These results highlight the fact that metal adsorption by MP can depend on the medium.

Different types of MPs can be found in the environment (Eerkes-Medrano et al., 2015; EU Commission, 2011; Frias et al., 2010; Wagner et al., 2014), all having different characteristics and different properties that can condition metal adsorption to MP. Therefore, different types of MP were tested (all in the same concentration): fishing line fibers (fibers), film MP (obtained from thin plastic bags), particles MP (obtained from bottle caps) and PE microspheres. Results showed that only film MP adsorbed Cu. In fact, soluble Cu concentration in solution was identical among the other types of MP, all being similar to that observed in control group (without MP). Such results may be associated with the fact that film MP has a higher surface of contact than the other types of MP, which probably allowed Cu adsorption. Although absorption of metals by MP has been previously reported (Aston et al., 2010; Holmes et al., 2012, 2014), to our knowledge, the comparison of metal absorption by different types of MP had not been done yet. However, Frias et al. (2010) showed that MP with different colours absorbs distinct levels of organic contaminants. The potential effects of colloids were less pronounced in this experiment, because Cu soluble concentration in solution slight varied over time and no significant differences between control and presence of PE were observed, probably due to aging of elutriate solution.

Plastic aging is another factor reported to affect MP capacity to adsorb metals, since in dynamic and natural conditions MP are subjected to alterations, namely, erosion, formation of functional groups in MP surface, adsorption and precipitation of different charged mineral and organic matter that confers heterogeneity and reactivity to MP surface (Aston et al., 2010; Holmes et al., 2012, 2014). In order to investigate the effect of aging, tests of possible adsorption of Cu to different types of MP were repeated but with aged MP. Aging of MP was carried out by putting each type of MP in a beaker containing estuarine water that was agitated by an orbital shaker for 2 weeks in a regular velocity. Results were similar to those observed for not aged MP. These results are contrary to those of Holmes et al. (2014), which observed a greater absorption of metals, including Cu, in aged MP pellets suspended in harbour for
8 weeks. The short period of aging process used in this study was probably not enough to induce pronounced changes on MP surfaces.

Holmes et al. (2014) observed that beached pellets absorbed greater amount of trace metals in river water than in seawater, which can indicate that salinity affects absorption of metals by MP. An experiment to test if salinity can influenced the absorption of Cu by MP was carried out, using as medium deionized water with different concentrations of NaCl. This constituted a simpler medium where, contrary to seawater or elutriate, there are no interferences of other compounds. Results indicate that salinity did not affect Cu absorption by MP. In fact, despite some variation over time, probably due to an initial adaptation of Cu to reach equilibrium in the solution (Brennecke et al., 2016; Holmes et al., 2014), no relevant differences in soluble Cu concentration in solution were observed at the end of the experiment. Bakir et al. (2014) also demonstrated that salinity had no influence in the sorption rate of POPs by microplastics. Although, conversely, the same study had concluded that sorption of POPs into sediments differs according to the levels of salinity present in the environment, and an increase of salinity may reduce POPs solubility, making them available to interact with sediments and buried plastics materials (Bakir et al. 2014). Therefore, salinity may not have a direct impact on metal absorption but it can influence indirectly MP capacity to absorb other contaminants.

After these preliminary tests, it was carried out an assay to test the impact of MP in phytoremediation. Although, preliminary results showed no adsorption of Cu by MP, there was still the hypothesis that the presence of the plant could change the capacity of MP to adsorb metals. On the other hand, MP could also block the roots preventing plants to accumulate Cu, which could cause nutrition problems for plants, since Cu, in low concentrations, is an essential element for plants (Rocha et al., 2014). PE was used as MP model due to their simpler handling and for being widely used in lab experiments. The interaction of microplastics with plants is a subject poorly investigated, although Bhattacharya et al. (2010) demonstrated that in laboratory conditions, freshwater algae Chlorella spp. and freshwater/saltwater algae Scenedesmus spp. absorb nanoparticles of polystyrene, having a decrease of the photosynthetic activity as consequence. Contrary, Davarpanah & Guilhermino (2015) concluded that the presence of polyethylene microsphere in the medium did not affect the average specific growth rate of Tetraselmis chuii. Recently, Gutow et al. (2015) showed
that the seaweed *Fucus vesiculosus* retains suspended MP on its surface, emphasizing the importance of studies investigating the impact of MP on plants.

This study showed that *P. australis* accumulated Cu in their belowground tissues, without relevant metal translocation. In fact, levels of Cu in roots and rhizomes of plants were considerably higher after exposure to medium doped with Cu relatively to field plants (control group), whereas Cu concentration in stems and leaves did not increase in comparison with non-Cu exposed plants. As expected, Cu accumulation was lower in the presence of sediments. These results are in agreement with previous studies that showed *P. australis* ability to phytoremediate sediments contaminated with Cu (e.g. Almeida *et al*., 2008, 2011, 2013; Silva *et al*., 2014). The presence of MP in the medium did not affect the absorption of Cu by plants, since results did not show significant variations on Cu content between plants exposed to Cu and to Cu+PE. Taking into consideration that, to our knowledge, there are no published studies reporting the interactions between MP and macrophytes, this study is the first to demonstrate that, in the selected conditions, MP did not interfere in the capacity of *P. australis* to phytoremediate Cu.

4.2. Impact of MP in phytoremediation of Cd by *P. australis*

All the experiments made with Cu were repeated for Cd. During all the preliminary tests, no adsorption of Cd by the MP was observed. As in experiments with Cu, no significant differences between Cd concentrations in elutriate solution with no aged film were observed. These results are in agreement with the observations made by Ashton *et al*. (2010), where Cd was not detected on new PE surface, after an experiment where PE pellet were suspended in harbour sea water for 8 weeks. The absence of Cd adsorption by aged MP may be related, as mentioned above for Cu, with the short period of the aging process. Nevertheless, it is important to notice that Cd capacity to link with organic ligands, as polymers of plastic and MP has been already documented by several studies (EU Commission, 2011; Holmes *et al*., 2012; Rochman *et al*., 2014). In the other hand, our results pointed that salinity might interfere with the adsorption of Cd.
Cd is a natural element relatively rare compared with other metals (Wedepohl, 1995), however is a priority substance under the Water Framework Directive legislation (Annex II, Directive 2008/105/EC), due to its high toxicity at low concentrations. Cd is a not an essential element for plants that can interfere negatively on plants growth and development, however plants are provided with internal mechanisms of defense against contaminants that involves, among other things, the uptake and accumulation of metals (Almeida et al. 2011; Benavides et al., 2005). The levels of Cd that are taken up by plants depend on several factors, namely temperature, pH, redox potential, concentration of other elements and presence of organic matter. All these factors modulate the bioavailability and concentration of Cd. In that sense, the present study investigated if the presence of MP in the medium could alter the uptake levels of Cd by *P. australis*.

In the assay with plants, results showed that, similarly to the assay with Cu, *P. australis* accumulated Cd in their belowground tissues, without significant metal translocation. Again, the levels of Cd in roots and rhizomes were higher after exposure to medium doped with Cd, relatively to field plants, while levels of Cd in stems and leaves did not increase in comparison with non-Cd exposed plants. Similarly to Cu, the presence of sediments in the medium resulted in lower Cd accumulation. These results are in agreement with previous studies that showed *P. australis* ability to phytoremediate sediments contaminated with Cd (e.g. Almeida et al., 2008, 2011, 2013, Silva et al., 2014). In the case of Cd, the presence of MP in the medium also did not affect the absorption of Cd by plants, since no significant variations on Cd content between plants exposed to Cd and to Cd+MP were observed. So, in the present conditions, similar to Cu, no significant effect of MP on plants Cd phytoremediation potential was also observed.
Final remarks

After being released or fragmented in the environment, MP can be dragged by the wind or rain from land to surface waters, being transported to freshwater and seawater. Once in the aquatic environment, MP can float or be submerged depending on their density.

Most of the commercialized plastic, including PE, are buoyant in seawater (Duis & Coors, 2016). However, MP density may be increased by several factors, as aggregation of organisms on their surface or erosion (Morét-Ferguson et al., 2010), which may lead to the sinking of microplastics. In fact, Browne et al., (2010) and Weinstein et al. (2016) observed that MP tend to be composed of denser plastics, additionally, Browne et al., (2010) also observed that MP are more abundant at the mouth of the river. That leads to an accumulation of MP in the sediments since the current available data indicates that degradation of plastic debris in the environment is an extremely slow process (Duis and Coors, 2016; Weinstein et al., 2016). Thus, MP become part of the sediment, both on the beach and on the sea bottom. Currently it is unclear how the environment and wildlife respond to exposure to MP (EU Commission, 2011).

With this work, MP potential to interfere on phytoremediation of Cu and Cd were not demonstrated, neither the capacity of MP to adsorb metals. Significant differences on metal concentrations were only observed in elutriate solutions with MP of film, which can be related with the fact that film MP have a higher surface area comparing with other types of MP, that probably allowed the adsorption of metals.

As future perspectives, there is the intent to do more research regarding the capacity of film MP to interfere with phytoremediation of metals by salt marsh plants.
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