

Master on Bioengineering

Bioremediation as nature based solution for emerging contaminants removal

Patrícia Rodrigues da Cunha

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Supervisor at the University: Dr. C. Marisa R. Almeida

Co-supervisors: Dr. Ana Paula Mucha and Dr. Joana Fernandes

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Abstract

The increasing number of world population has led not only to an excessive use of several natural resources but also to the uncontrolled discharge of several substances to the environment. Some of these substances are already known for their potential negative effect on human and animal life; others are only now being studied. Emerging contaminants, such as pharmaceuticals, are receiving increasing attention from environmental regulators to understand their potential adverse effects on organisms.

Estuaries are increasingly affected by all the types of pollutants as it suffers from the pressure of large urban areas and all the effluents that derive from its human vicinity. As important ecological areas, it is relevant to find strategies to fight the adverse effects posed by numerous contaminants and eventually recover the affected environments.

This study aimed to evaluate the bioremediation potential of an estuarine salt marsh plant for the removal of emerging contaminants from an estuarine environment. Initially, the presence of several metals and two pharmaceuticals in non-vegetated sediments, sediments vegetated with three different salt marsh plants, and in the different plant parts (only metals) was monitored *in situ* in three different time periods, as the presence of multiple contamination can affect ecosystem recovery. A controlled laboratory experiment was also assembled to evaluate the potential of *Phragmites australis* and its native microbial community in the removal of bezafibrate and paroxetine. Additionally, a bioaugmentation treatment was performed to observe if it would enhance the removal potential of the previous treatment.

In situ survey indicated that none of the metals monitored was registered at concentrations higher than the legal limit which allowed to classify the sediments as clean. Besides, in general vegetated sediments presented higher metal levels than non-vegetated sediments, highlighting salt marsh plants potential for pollutants removal/retention. Neither bezafibrate nor paroxetine were detected in any of the sediments collected.

Regarding the experiments assembled in laboratory, in both groups with and without bioaugmentation treatment, bezafibrate and paroxetine were equally highly removed indicating that the system had capacity for natural attenuation of both compounds. The microbial consortium obtained from estuarine sediments also revealed potential to remove both pharmaceuticals completely at the end of two-week cycles of exposure. Overall, the selected salt marsh plant and the microorganisms associated with its rhizosphere showed potential to be used as a bioremediation tool for the removal of pharmaceuticals from estuarine environment, contributing for the recovering of these ecosystems.

Keywords: bioremediation, estuaries, emerging contaminants, metals.

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

4th July 2022

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Notation and Glossary

API	Active Pharmaceutical Ingredient
BZF	Bezafibrate
ECs	Emerging Contaminants
HPLC	High Performance Liquid Chromatography
LMAs	Lipid Modifying Agents
MM	Mineral-salt Medium
PRX	Paroxetine
SPE	Solid Phase Extraction
SSRIs	Selective serotonin reuptake inhibitors
WWTPs	Wastewater treatment plants

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1 Introduction

1.1 Framing and presentation of the work

The worlds' population has increased seven-time fold since 1800, reaching 7.9 billion people in 2022 (Roser, Ritchie et al. 2013). This continued growth, the high quality of life and consequent higher demand from the consumers has been causing the excessive use of several resources and environmental issues like never before. Recently, several substances, either from anthropogenic or natural sources, have been detected in water bodies and are classified as emerging contaminants (ECs). These products can be divided into different categories such as pharmaceuticals, personal care products, industrial chemicals and steroid hormones, among others (Gomes, Simões et al. 2018). ECs are discharged to the environment through toxic waste disposal, incorrect waste of chemical products, sludge disposal, or use of fertilizers, ending up in water bodies (Khan, Naushad et al. 2022). Wastewater discharges from municipal and hospital sewage, treated and untreated, as well as effluents from the contaminants' production sites are the main sources of emerging pollutants (Fatta-Kassinos, Meric et al. 2011). The effects of these substances in organisms, animals or humans are still not fully comprehended but have the potential to significantly harm them, depending on the quantity and time of exposure.

A review of the World Health Organisation in 2012 on the occurrence of pharmaceuticals in drinking and surface waters has shown that the concentration of pharmaceuticals in surface and groundwater sources influenced by wastewater discharges are usually less than $0.1 \mu\text{g L}^{-1}$ (World Health Organization 2012). Bezafibrate (BZF) is a pharmaceutical that belongs to the fibrates' family, used to lower lipids levels. Its mechanism of action consists in the activation of a receptor involved in breaking down dietary fat, accelerating this process. Lipid modifying agents (LMAs), where bezafibrate is included, have registered an increase in its consumption from 2008 to 2018 with an annual rate of 4.13 %. Fibrates are the second most used class of LMAs and Portugal is in the top three countries with the highest consumption of these pharmaceutical products in 2018 (Blais, Wei et al. 2021). Hence, it comes as no surprise that this product has been detected in surface waters in European countries such as Austria, Finland, France, and Germany, with a maximum concentration in the last one of $3.1 \mu\text{g L}^{-1}$ (World Health Organization 2012). Also in Portugal, BZF was detected in surface waters at a mean concentration of 13.69 ng L^{-1} (Pereira, Silva et al. 2017). Paroxetine (PRX) is a drug used in the treatment of major depressive and panic disorders, fitting in the category of selective serotonin reuptake inhibitors (SSRIs) (Hwang, Kim et al. 2021). In 2015, antidepressants like PRX accounted for 3.7% of all the drugs sold in Portugal, a value only surpassed by Canada and

Spain (Estrela, Herdeiro et al. 2020). A study analysed the presence of several antidepressants in the influent and effluent of multiple wastewater treatment plants (WWTPs) across Portugal and PRX was detected with mean concentrations of 169.97 and 81.1 ng L⁻¹, respectively (Silva, Pereira et al. 2014). WWTPs are not prepared to completely remove these contaminants, especially since they are present in low concentrations, and they end up being discharged to water bodies, that can reach estuaries, for instance, where its persistence and bioaccumulation can be toxic to several organisms (Rout, Zhang et al. 2021).

Estuaries and the wetlands within them include bodies of water in the areas where rivers meet the sea. Their unique characteristics of the mixture of freshwater and salty seawater provide the unique conditions for the development of specific plants and animal communities. In wetlands, the soil is poorly aerated and water saturated, compatible with the growth of vegetation adapted to wet conditions (Ansola, Arroyo et al. 2014). The exaggerated urban development around coastal areas due to rapid population growth has negatively impacted estuaries, with domestic and industrial discharges compromising the health and viability of their communities (Vasconcelos, Reis-Santos et al. 2007). These areas display crucial roles in nutrient cycling, erosion control, pollutants removal and sediment accretion, therefore it is relevant to study them and its properties and strategies for its protection and recovery.

Conventional physical and chemical methods to clean contaminated sites are widely used and present high efficiencies. However, they can be of high cost, some of them are limited to small areas, may require additional land for waste storage, and are dependent on the type of sediment present (Li, Zhou et al. 2019). Bioremediation comes up as a green technology for pollution removal via biological processes. Bioremediation is the use of microorganisms and/or plants in the treatment of contaminated locations, being a sustainable remediation technology to reset the natural conditions of contaminated soils and water (Chibuike and Obiora 2014, Dixit, Wasiullah et al. 2015). These organisms are able to detoxify or degrade ECs into less dangerous forms through their metabolic pathways, using it as sources of energy and carbon (Jabbar, Salih et al. 2022). This technology presents itself as a more eco-friendly, non-invasive, cheaper, and more definitive solution than conventional methods (Dzionek, Wojcieszńska et al. 2016).

This study aims to explore the use of a salt marsh plant to remove BZF and PRX from estuarine sediments.

Initially, a survey to evaluate the presence of BZF and PRX in different sediments, non-vegetated and vegetated by different salt marsh plants (*Juncus maritimus*, *Phragmites australis* and *Spartina maritima*) was carried out in three different time periods (August 2021, November 2021, and February 2022) in different locations of the Lima River estuarine salt

marsh area. The objective was to evaluate the potential of plants and associated microorganisms to remove BZF and PRX. As the presence of different pollutants might influence removal and recovery processes by saltmarsh plants and their associated microorganisms, different metals were also determined in the collected sediments and salt marsh plants.

Afterwards, controlled experiments were carried out to determine if an enriched estuarine sediment slurry, previously exposed to the selected pharmaceutical compounds, would improve the removal of the BZF and PRX from a simulated contaminated estuarine environment. The objective was to assess the systems' bioremediation potential.

1.2 Organization of the thesis

This thesis consists of six chapters, starting with the Introduction (Chapter 1), where an initial framing and presentation of the work can be found, as well as its organization.

Chapter 2 consists of Context and State of the Art, in which a more detailed explanation of the problem that drives this study is given, as well as the topics approached in this thesis, such as emerging contaminants, analysed pharmaceuticals, bioremediation and bioaugmentation, estuaries and salt marsh plants, among others.

The materials and methods used to perform the work is described in Chapter 3.

The results and their discussion are presented in Chapter 4 for the Lima River estuary *in situ* survey and Chapter 5 for the laboratorial experiment.

Chapter 6 sums up the major conclusions gathered from the developed study and assesses the work done, identifying the objectives that were achieved and future work that still needs to be done.

2 Context and State of the art

Water covers approximately 71% of the earth's surface. However, only 3% of that amount is freshwater and 2.5% is unavailable, froze in glaciers, in the atmosphere or soil, highly polluted or too deep into the earth's surface to be extractable at a reasonable cost. Life as we know it is only possible due to the existence of liquid water and, since available drinking water represents such small percentage of the freshwater, the conservation of these water resources is of extreme importance.

2.1 Emerging Contaminants

Emerging contaminants (ECs) are defined as synthetic or naturally occurring substances that, when present in the environment even at trace concentrations, can potentially cause adverse reactions in humans and organisms, and are not commonly monitored (Rosenfeld and Feng 2011).

The global awareness for pollutants began with the publication of the book "Silent Spring" in 1962 (Carson 2002), which indicated that the widespread use of DDT with the goal of eliminating mosquitoes and other pests had contributed to the disappearance of many birds. However, due to the lack of analytical methods sensitive enough to detect low levels of ECs, more focused research on the topic began only twenty years ago. In 2002, a US survey on water quality detected several pharmaceuticals and hormones in many US streams, raising the alarms for the issue of ECs (Kolpin, Furlong et al. 2002).

The categories of ECs are varied: pharmaceuticals, personal care products, hormones, illegal drugs, cleaning products and other industrial chemicals (Rathi, Kumar et al. 2021). These compounds find their way to the environment through municipal WWTPs discharges, effluents from hospitals and industries, animal agriculture and aquaculture, and incorrect disposal of contaminated products. The destination are bodies of water and these substances have been detected all over the world in WWTPs effluents, surface water, drinking water and groundwater (Noguera-Oviedo and Aga 2016).

In WWTPs, the influent is submitted to several treatments to remove the organic load, larger particles and debris, and other substances that may be present. Activated sludge is one of the most common treatments used worldwide and is a process with high concentration of microorganisms, especially bacteria, protozoa and fungi, kept in suspension by stirring, to remove organic matter from wastewater (Rajasulochana and Preethy 2016). Although working perfectly for the removal of organic matter, biodegradation of ECs does not occur with the same efficiency, since is dependent on many factors such as physical-chemical properties of

the substances, carbon load, hydraulic retention times, redox conditions, and the microbial community itself, among others (Noguera-Oviedo and Aga 2016). ECs escape this removal process and end up in WWTPs effluents and, eventually, rivers, oceans and even drinking water sources.

Pharmaceuticals are one of the main categories of ECs and are defined as biologically active compounds used in the prevention, cure, and treatment of diseases. They can be formed into a pill or a liquid and consist of one or more active pharmaceutical ingredients (APIs), excipients, and additives. APIs present different physicochemical and biological properties and, changes in their chemical structure can have a drastic impact in their environmental fate (Cunningham 2004). The usage of this type of products is expected to increase due to the extended life expectancy, world population growth and higher life standards (Kümmerer 2010). Monitoring these substances in the environment, namely surface waters and urban wastewater, is not only difficult but also time-consuming mainly due to the complexity of the samples' matrices and diverse physicochemical properties of the compounds (Szymańska, Wiergowski et al. 2019). Hence, continuous monitoring of the presence of these analytes in water is, in most cases, not easily feasible.

Pharmaceuticals can be classified according to their therapeutic category such as antibacterials, antidepressants and cardiovascular agents, where lipidic agents are included (US Food and Drug Administration 2018). Portugal is one of the countries with the highest antidepressant consumption rates registering a consistent increase in its usage since 2000 (Estrela, Herdeiro et al. 2020). Between 2010 and 2020, the number of packages of antidepressants dispensed in pharmacies increased 83.5%, and SSRIs, such as PRX, were the most prescribed (Martins 2021). A higher number of patients are being diagnosed with depression which could explain the increased prescription of these type of substances. Portugal is also pioneer in the consumption of lipid modifying agents, being BZF one of the most used (Blais, Wei et al. 2021).

A study to assess the occurrence of pharmaceuticals in Portuguese surface waters was performed, revealing the presence of the substances in 27.8% of the samples. SSRIs had the highest detection frequency, 27.8%, and an average concentration of 37.9 ng L⁻¹, whereas LMAs were detected in 8.33% of the samples, registering a mean concentration of 9.47 ng L⁻¹ (Pereira, Silva et al. 2017).

2.2 Pharmaceuticals occurrence in the environment

Since the human/animal body does not completely absorb or metabolize the administered substances, they end up being excreted through urine and faeces that are directed to WWTPs, turning these facilities into a sink of pharmaceuticals (Phonsiri, Choi et

al. 2019), at the same time as it becomes a source to the aquatic environment. Pharmaceuticals administered in veterinary medicine are excreted by the animals and can end up in the soil. If they are not bound to soil constituents, they can reach groundwater or, in case of heavy rain events, they can be washed and reach large bodies of water (Kümmerer 2010).

Several studies reported that, although not expected, the emissions from manufacturing and production facilities can also be a relevant source of pharmaceuticals to the environment (Larsson, de Pedro et al. 2007, Thomas and Langford 2010). For obvious reasons, hospital wastewaters register higher pharmaceuticals concentrations than municipal wastewater (Kümmerer 2000, Kümmerer 2010). However, the load of pharmaceuticals in hospital effluents is much lower than municipal effluents since most of the usage occurs in the households (Kümmerer 2010).

Unproper disposal of leftover or expired medicine, often discarded down the drain or toilet (Bound and Voulvoulis 2005), also contributes to the pharmaceutical pollution of households wastewater. If the unused drugs are discarded via household waste and afterwards put in a landfill, the pharmaceuticals will eventually end up in the effluent of the landfill and, if this is not collected and properly treated, it can be a source of contamination for surface and groundwater (Kümmerer 2010).

Legislation regarding the legal limits of pharmaceuticals in wastewaters prior to discharge are very scarce. World Health Organization indicates that pharmaceutically active compounds should not be discharged into wastewater. Hospital wastewaters discharge has no specific standards in European countries about the limits of ECs in these effluents (Yan, Zhang et al. 2020). Many countries consider hospital wastewater similar to municipal wastewater and therefore is discharged without any pre-treatment to the municipal sewer system. In fact, guidelines and legal limits are usually posed to effluents of WWTPs and they concern the basic physicochemical characterization such as pH, temperature, chemical oxygen demand, biochemical oxygen demand and total suspended solids (Yan, Zhang et al. 2020). However, the concentration of pharmaceuticals in hospital wastewaters can also be relevant and this factor is not being considered in the majority of hospital effluents treatment.

The European Commission has elaborated a Watch List, reviewed from time to time, containing emerging contaminants that should be monitored in the environment, in which, among them, are several pharmaceuticals (Commission Implementing Decision /495 2015). Along with trying to reduce the consumption of pharmaceuticals to contain the problem at its source, the European Commission also aims to improve the waste management system and reduce the waste of these substances to lower the risk of their appearance in the environment. Furthermore, advanced wastewater treatment processes are necessary to

efficiently remove pharmaceuticals from effluents and a continuous monitoring of these substances in several environments is of extreme importance to understand their eco-toxicity and fate in the environment (Khasawneh and Palaniandy 2021).

2.2.1 Pharmaceuticals in Portuguese rivers and estuaries

The presence of pharmaceuticals in Portuguese waters has been studied in recent years. Samples collected in Mondego, Tagus and Guadiana estuaries revealed the presence of BZF at a mean concentration of 13.69 ng L^{-1} but its prevalence was only in 2.78% of the samples analysed (Pereira, Silva et al. 2017). Samples from Leça river were analysed for a set of 24 pharmaceuticals, among them BZF, which was present at a maximum concentration of 770 ng L^{-1} (Gonçalves, Sousa et al. 2013). In another study, in Antuã and Cértima rivers, the concentration of BZF in water was always under 10 ng L^{-1} , whereas in the Leça river the maximum concentration registered was 40 ng L^{-1} (Barbosa, Ribeiro et al. 2018). A study conducted in the Tejo estuary revealed the presence of BZF in 87.1% of the analysed samples, with a mean concentration of 3.31 ng L^{-1} (Reis-Santos, Pais et al. 2018).

Regarding the presence of PRX in surface waters, this compound was detected in Zebro river, Guadiana basin, with a mean concentration of 60.65 ng L^{-1} (Palma, Fialho et al. 2020). In the Lis river, among other substances, PRX was detected at a mean concentration of 25.5 ng L^{-1} (Paíga, Santos et al. 2016).

Although the studies on the presence of the selected pharmaceuticals in Portuguese rivers sediments are scarce, the ones that exist revealed the presence of both compounds. Lima river has been studied for the presence of BZF and PRX in sediments, indicating their presence in rhizosediments at concentrations of 0.14 and $0.19 \text{ } \mu\text{g g}^{-1}$ (Dias 2018). Also in Cávado river sediments these pharmaceuticals were detected in low concentrations, $0.35 \text{ } \mu\text{g g}^{-1}$ for BZF and $0.16 \text{ } \mu\text{g g}^{-1}$ for PRX (Dias 2018). Other pharmaceutical compounds were detected in both sediments and water of Douro and Leça rivers (Paíga, Santos et al. 2016), which indicates the relevance of monitoring these compounds in estuaries.

An estuary is an open body of water that receives both saline water from the sea and freshwater from the rivers, generating different levels of salinity. This feature gives estuaries unique properties and leads to their acknowledgement as distinct habitats. Apart from salinity, a gradient of sediment size, alterations in turbidity and chemical composition across the water column are also present (Elliott and McLusky 2002).

Commonly known as places for recreational activities and general enjoyment, these locations are also relevant for many other reasons. Most evidently, estuaries have particular characteristics that turns them into critical natural habitats. Many animals, such as birds and fish, depend on estuaries to live, feed, migrate, and reproduce. Moreover, estuaries act as a

cleaning agent since the water it receives is filtered through their sediments and salt marshes, allowing for the removal of nutrients and other pollutants, generating cleaner water for all the living organisms.

Since estuaries are areas naturally rich and that favour the development of numerous economic activities, they suffer from the urban pressure caused by the growing population living around these areas. Agriculture, animal farms, industries, aquaculture, ports and urban areas are the main factors that contribute to anthropogenic pressure in estuaries, altering the ecosystems' dynamic (Newton, Icely et al. 2020). Furthermore, estuaries are receivers of industrial and municipal wastes, as well as direct sewage disposal. Frequently, these wastes are contaminated with several compounds, namely pharmaceuticals, and the continuous exposure of the organisms to these pollutants can have a long term impact on ecosystems' health (Kennish 1994), due to changes in the organisms' systems, either digestive and/or reproductive, which implicate with the the food chain.

2.3 Studied Pharmaceuticals

Bezafibrate is an antilipemic agent that decreases triglyceride levels and total and low density lipoprotein (LDL) cholesterol levels and increases high density lipoprotein (HDL) cholesterol levels. It belongs to the family of lipid modifying agents (National Center for Biotechnology Information 2022).

Paroxetine is a benzodioxol, highly potent and selective 5-HT uptake inhibitor that binds with high affinity to the serotonin transporter. Therefore, it works as a selective serotonin reuptake inhibitor, used in the treatment of depression, anxiety disorders and obsessive-compulsive disorder (National Center for Biotechnology Information 2022).

Their chemical and physical properties are summarized in Table 2.1.

Table 2.1 - Chemical and physical properties of bezafibrate and paroxetine.

	Bezafibrate	Paroxetine
Molecular formula	$C_{19}H_{20}ClNO_4$	$C_{19}H_{20}FNO_3$
Molecular weight (g mol ⁻¹)	361.8	329.4
Log K _{ow}	4.25	1.32 (pH=7)
Water solubility	1.131 mg L ⁻¹ at 25° C	1.55 µg mL ⁻¹ at 37° C

2.4 Techniques for pharmaceuticals detection and quantification

The major challenge regarding the presence of pharmaceuticals in the environment is the development of analytical techniques capable of identify and quantify the target compounds at trace levels, in a fast and efficient way.

Gas chromatography was a technique developed to separate molecules with similar characteristics, in a rapid and reliable way. In this case, gas is the mobile phase and the stationary phase is either solid or liquid. Its very high selectivity and resolution, as well as good accuracy, precision and high selectivity, made it a very popular technique in the identification of widespread pollutants such as volatile organic compounds, polycyclic aromatic hydrocarbons, pesticides, and halogenated compounds (Santos and Galceran 2002). Shortly after, gas chromatography coupled with mass spectrometry became the most common technique to analyse environmental samples. This technique joined the separation efficiency of gas chromatography and the high sensitivity and great qualitative information of mass spectrometry (Santos and Galceran 2003).

High performance liquid chromatography (HPLC) came as a quantitative analytical tool which can also provide a qualitative analysis of samples. The use of very small particles for the solid absorbent in the stationary phase allowed to upgrade the resolving power, detection, and quantification, while the time required to analyse the sample was reduced. Once this technology was fully developed, it proved to be much more adequate for the analysis of pharmaceuticals than gas chromatography (Lough and Wainer 1995).

The analysis of environmental samples is a complex task since the target compounds may be present in extremely low concentrations and in very complex matrices. HPLC revealed to be insufficient to unequivocally detect the target compounds. Taking this in consideration, liquid chromatography coupled to tandem MS/MS emerged as the new technology that provides the selectivity and sensibility required to qualify and quantify several ECs (Farré, Kantiani et al. 2012).

Recently, quadrupole time of flight tandem mass spectrometers have been coupled with liquid chromatography based ionization techniques for the analysis of trace elements since this combination allows to achieve the accurate mass measurements and improves selectivity (Farré, Kantiani et al. 2012).

Since the target substances are present in the environmental samples at very low concentrations and in highly complex matrices, solid phase extraction (SPE) is a step utilised in sample preparation. The most common procedure consists of passing the sample through a porous column (cartridge) filled with a specific sorbent to retain the target compound, eluting it afterwards with a small amount of solvent. SPE allows the preconcentration of the

analyte to improve detection limit and simplifies the matrix by eliminating interferences from the sample (Faraji, Yamini et al. 2019).

2.5 Bioremediation

With the uprising concern regarding the discharge of contaminated effluents in the environment, there has been an effort in restoring the locations already affected by different pollutants, including ECs. Several chemical and physical methods are already used in the treatment of contaminated water bodies and soils, especially for the removal of metals (such as chemical precipitation, filtration, oxidation or reduction, ion-exchange, reverse osmosis, membrane technology, among others) (Dixit, Wasiullah et al. 2015) or organic pollutants (such as advanced oxidation processes namely photocatalysis, Fenton and ozonolysis, which can be combined with sonolysis) (Sathishkumar, Mangalaraja et al. 2016). However, these methods can be inefficient or very expensive when concentrations are very low.

Bioremediation is defined as a technique that takes advantage of microbial processes, plants, or a combination of both for the treatment of contaminated locations (Gouma, Fragoeiro et al. 2014). This method is widely used for the treatment of contaminated soils since it is perceived as a natural process and is more cost-effective due to its application on site, eliminating transportation costs (Chibuike and Obiora 2014). Phytoremediation refers to the use of plants and associated microorganisms in the removal of different contaminants, such as organic pollutants and metals, from diverse matrices like sludge, soil, surface water and wastewater. This method has the sun as energy source and is applied *in situ*, reducing the overall costs, and is an eco-friendly alternative to physicochemical technologies (Dixit, Wasiullah et al. 2015). Plants have different remediation strategies, represented schematically in Figure 2.1, that can be used simultaneously. Phytodegradation is the metabolization of organic contaminants inside the plant cells by specific enzymes. Metals can be absorbed by the roots and transported to other plant parts, where they are accumulated, in a process called phytoextraction. On the other hand, metals can suffer the action of roots exudates and precipitate, being trapped in the soil matrix. This process of phytostabilization can also be applied to both organic and inorganic contaminants that are incorporated into the cell wall. Phytovolatilization is the ability that some plants possess to absorb certain contaminants by the roots and convert it to non-toxic forms, releasing it from their leaves to the atmosphere. Lastly, phytostimulation refers to the proliferation of microorganisms, specific of the rhizosphere, as a result of the use of roots exudates and metabolites as sources of carbon and energy, which can contribute to the degradation of organic pollutants (Favas, Pratas et al. 2014).

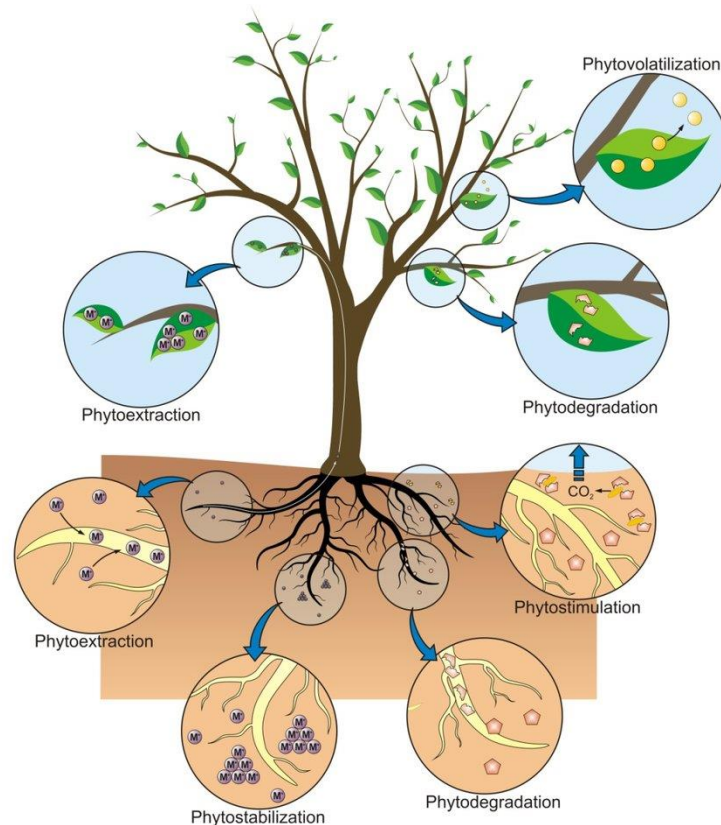


Figure 2.1 - Schematic representation of phytoremediation strategies (Favas, Pratas et al. 2014)

Microorganisms are present everywhere, from soil to water, and therefore, they always come in contact with the contaminants present in the environment. Some microbes have the capacity to breakdown the pollutant via their metabolic pathways, ending up degrading it completely or accumulating it. Others can only transform the pollutants into less toxic substances, that will not have such adverse effect in the environment. The use of microorganisms as bioremediation technology allows for the treatment *in situ*, being a low cost technology compared to other techniques that involve transferring the contaminated matrices from one place to another. As it is a natural process, the public accepts it more easily. However, it is a process that requires more time to achieve the desired results and some contaminants may not be removed by this technique or even be transformed to more hazardous compounds (Tekere 2019).

Autochthonous or allochthonous organisms can be added to the contaminated site to enhance the removal of the pollutants. Such process is named bioaugmentation and the organisms can be genetically modified to degrade a specific compound, enhancing the compound's degradation rate. However, the success of this technique depends on the organisms' ability to adapt to the contaminated environment and to compete with the indigenous microbial community. Other factors can influence their adaptation to the site such

as pH, temperature, nutrient content, organic matter content, humidity and aeration (Tribedi, Goswami et al. 2018).

Instead of adding a new microbial consortium to the site, the present microbial community can be stimulated. Biostimulation refers to the addition of specific key nutrients such as phosphorous, nitrogen, or electron donors to stimulate the existing organisms into a more efficient and rapid removal of the pollutants. Moisture content, pH and temperature are the few environmental parameters that can influence biostimulation (Tribedi, Goswami et al. 2018).

Bioremediation has already been successfully applied in different contexts such as in sludge of an oil refinery (Roy, Dutta et al. 2018), crude-oil contaminated soil (Xu and Lu 2010), soils and water contaminated with dichloroethenes (Olaniran, Pillay et al. 2006), and polyurethane buried in soil (Cosgrove, McGeechan et al. 2010). Regarding ECs, research has shown the potential of some plants to remove veterinary antibiotics such as tetracycline and sulfadimethoxine (Tasho and Cho 2016). Another study reported the removal of metals and antibiotics from a sewage effluent when a specific strain of *Bacillus subtilis* was present (Al-Gheethi, Lalung et al. 2015). Microalgae has shown its potential for the bioremediation of nutrients from wastewater streams but only recently have caught the attention for its use in the removal of ECs, either by bioadsorption or bio uptake (Sutherland and Ralph 2019).

As previously stated, estuaries are one of the most affected ecosystems regarding ECs contamination. Therefore, it is relevant to understand how native plants and organisms react to their presence and if they have a bioremediation capacity. Several plants have shown to be tolerant to pharmaceuticals, henceforth the use of wetland plants as bioremediation strategy to remove ECs seems to be the most environmentally friendly approach (Rai 2018). In laboratory conditions, the salt marsh plant *P. australis* together with estuarine sediment could remove 47% and 90% of the BZF and PRX present in the solution, respectively (Dias, Correia et al. 2020). Several bacterial strains have also been identified as potential bio degraders of BZF and PRX and, upon further developments, can be used as a tool to restore contaminated locations (Fernandes, Duarte et al. 2020). To confirm the applicability of both phytoremediation and bioremediation in estuarine environment, experiments should be carried out with conditions closer to the real environment as possible. Moreover, it is relevant to consider the presence of other pollutants, such as metals, since this may influence the remediation potential performed by the microorganisms. Taking this in consideration, phyto and bioremediation techniques should be studied for the removal of pharmaceuticals in estuarine areas.

3 Materials and Methods

3.1 Sample collection and preparation for determination of metals and pharmaceuticals

Juncus maritimus (Lam.), *Phragmites australis* (Cav.) Trin ex. Steud., and *Spartina maritima* (Brongn.) were collected at low tide in August 2021 (first sampling), November 2021 (second sampling) and February 2022 (third sampling), at different sites in Lima River estuary (NW Portugal). The sampling sites are identified in Figure 3.1.

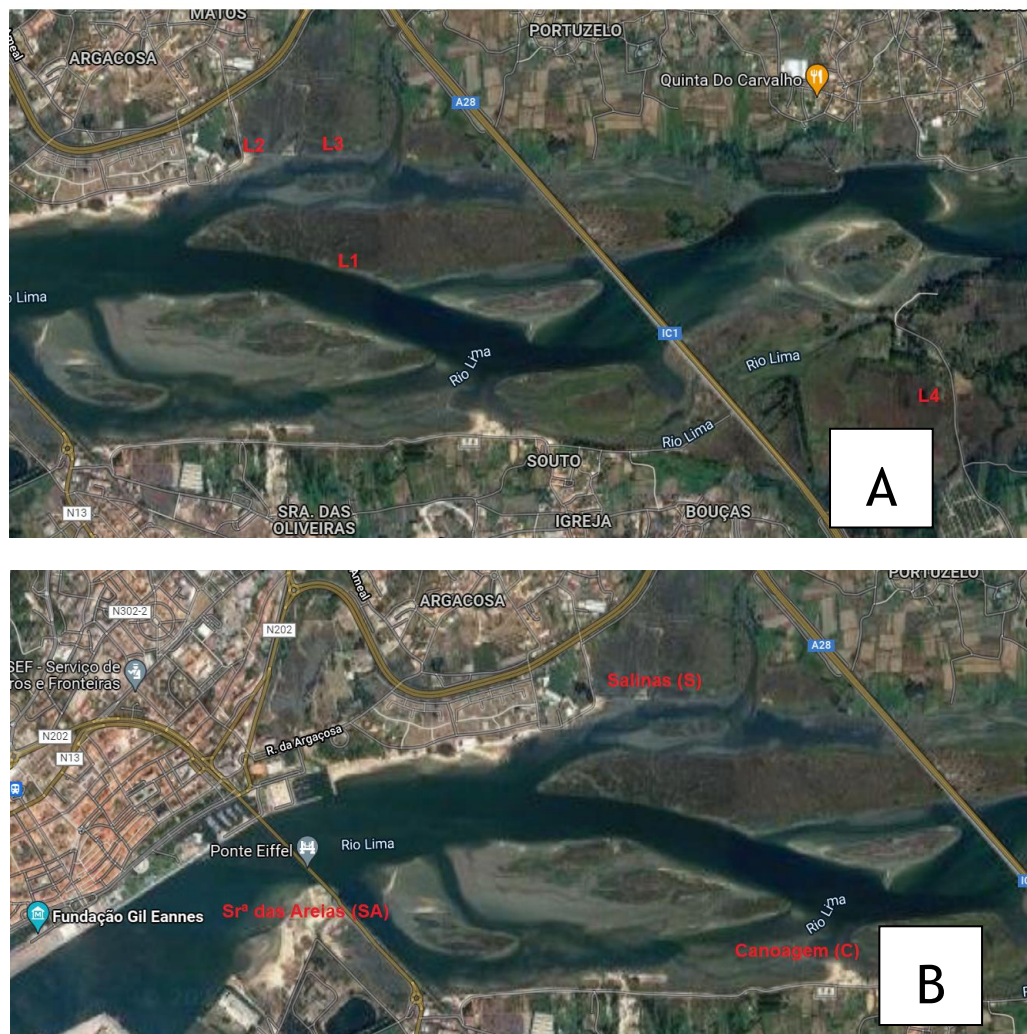


Figure 3.1 - A: First sampling locations, L1 colonized by *J. maritimus* and *S. maritima*, L2 colonized by *J. maritimus* and *P. australis*, L3 and L4 colonized by *J. maritimus*; B: Second and third sampling locations, Salinas and Srª das Areias colonized by *J. maritimus* and *P. australis* and Canoagem colonized by *J. maritimus* and *S. maritima*.

The sampling campaign in August was a preliminary sampling to evaluate possible sampling sites. After that, new sites were selected, and two other sampling campaigns were carried out.

Each collected sample was put into an individual plastic bag using metallic shovels, for rhizosediment (sediment in contact with plants belowground tissues) and non-vegetated sediment, or plastic gloves, for plants, and was immediately carried to the laboratory.

On arrival at the lab, plants were thoroughly rinsed with deionized water, placed in trays, covered with filtering paper to avoid contamination, and put to dry at room temperature until constant weight. Sediments and rhizosediments were individually prepared and dried as the plants for metal analysis. Afterwards, sediments and rhizosediments were homogenized and sieving through nylon nets of 2mm to remove large stones and dead roots. Plants were separated into stems, rhizomes (when present), and roots which were separately cut and homogenized. Vegetated and non-vegetated sediments were frozen at -20 °C and lyophilized afterwards for pharmaceuticals determination.

3.2 Evaluation of bioaugmentation coupled with *P. australis* bioremediation potential

3.2.1 Sample collection and experimental assembly

P. australis and respective rhizosediment were collected in the Lima River estuary (Sr^a das Areias) during low tide on the 2nd of May 2022. When arriving to the lab, the plants and its sediment were placed inside a vase lined inside with aluminium foil to prevent the adsorption of pharmaceutical compounds. A layer of pebbles was placed on the bottom to allow water circulation, minimize sediment loss, and prevent clogging. A tap was inserted at the bottom of each vase to drain the water when necessary. Plants were watered every two days with Hoagland nutrient solution until the beginning of the experiment. The system was kept inside of an aerated greenhouse, as observed in Figure 3.2.

A portion of sediment was kept at - 20°C for pharmaceutical analysis and considered as time zero (T₀) samples. Aerial part, roots and sediments were also analysed for metal content. Another portion of sediment was collected into sterile bags and kept at -20°C for microbial community characterisation.

In total, 15 vases were setup with 5 different groups (each in triplicate): control; control doped with BZF; control doped with PRX; bioaugmentation with BZF enriched slurry; bioaugmentation with PRX enriched slurry. Once the experiment started, the vases were watered every two days with 100x diluted Hoagland nutrient solution adjusted to 10% NaCl (similar to estuarine environment) doped with the respective pharmaceutical at a

concentration of $100 \mu\text{g L}^{-1}$. This concentration was used in previous studies where *P. australis* was used (Dias, Correia et al. 2020) and, although is higher than the ones found in river waters, it simulates a worst case scenario where plants are exposed to great stress. The volume of water used was to keep the system moist, simulating what happens in estuaries with tide changes.

The first cycle started on May 30th and on the day after 100 mL of slurry, adjusted to 10% NaCl, from the respective microbial consortium (see below) was added to the vases representing the bioaugmentation treatment. Additionally, 100 mL of saline solution was also added to the remaining vases so that the volume of water added remained the same. Sediments samples were collected from each vase prior to the first doping for microbial and pharmaceutical analysis.



Figure 3.2 - Experimental setup.

Cycles of 15 days were performed where prior to new slurry addition, sediments samples were again collected from each vase for pharmaceutical and microbial community analysis. On watering days, any remaining water in the vases was collected for pharmaceutical concentration determination.

3.2.2 Sampling and microbial consortium setup

The *P. australis* rhizosediment used as inocula for the preparation of the enriched slurry (used in the bioaugmentation treatments) was collected in May 2022 in Sr^a das Areias site. Samples were collected in sterile containers and transported to the laboratory in refrigerated and dark conditions. Once in the laboratory, a portion of each collected sample was frozen at -20°C for microbial community analysis (future work) and the remaining was used to prepare the bacterial enriched slurry resistant to the each of the selected pharmaceutical.

For the slurry preparation, 50 g of homogenised sediment were put in 1 L sterilized flasks containing 200 mL of a mineral-salts medium (MM). Microcosms were supplemented with sodium acetate (500 mg L^{-1}) as a co-metabolite, to promote microbial metabolic activity. Microcosms were then doped with BZF or PRX (1 mg L^{-1}). For that, a proper amount of a methanolic standard solution (prepared by dissolving a known amount of each compound in methanol) of each compound was added to the medium. Microcosms were setup in triplicate for each pharmaceutical compound.

Microcosms (closed flasks) were incubated at room temperature (20°C), in the dark and in static conditions. This approach was based on a previous study designed to obtain a microbial consortium resistant to each of the pharmaceuticals (Duarte, Almeida et al. 2019). Experiments were carried out for cycles of 2 weeks with the first cycle starting on 18th of May. During each cycle, microcosms were fed with sodium acetate (500 mg L^{-1}) twice a week. To ensure the presence of oxygen in the microcosms, each microbial culture was transferred to a new sterilized flask once a week. At the end of each cycle, 100 mL of each microbial culture was transferred to a new flask containing 100 mL of MM and doped with the respective pharmaceutical compound in the same dosage.

To analyse pharmaceuticals removal and microbial community structure, 15 mL of the remaining culture was collected. The samples were centrifuged at 3046 rpm (VWR MEGA STAR 600R) for 15 minutes. The supernatant was collected for pharmaceutical analysis and the pellets were kept for community characterisation. The remaining volume collected from each triplicate (ca. 85 mL) was joined, homogenised, and added to the vases for bioaugmentation. At the end of the first and last cycles, the microbial community structure of this microbial consortium will be determined (future work).

3.3 Analysis of pharmaceuticals

3.3.1 Material and Reagents

To prevent contamination, all sampling and labware materials were soaked in 20 % (v/v) HNO_3 solution for at least for 24h, rinsed several times with bi-deionised water (conductivity $<0.1 \text{ mS cm}^{-1}$) and left to dry. All reagents used were pro analysis grade or equivalent.

3.3.2 Standard stock solutions

Initially 10 mg of BZF and 20 mg of PRX were weighed, then dissolved in 10 mL and 20 mL of methanol, respectively, giving a concentration of 1 g L^{-1} for each pharmaceutical. All these stock solutions were placed in amber vials and stored at -20°C , protected from the light.

3.3.3 Standard working and daily solutions

The standard working solutions were prepared from the individual standard stock solutions attaining concentration of 10 mg L^{-1} of BZF and of PRX. These solutions were also placed in amber vials and kept at -20°C . Standard daily solutions were prepared every day with concentrations between 0.2 and 5 mg L^{-1} . These daily standard solutions were prepared immediately before pharmaceuticals analysis in methanol and water solution (MeOH/H₂O 70:30 v/v).

3.3.4 SPE extraction

SPE was performed to clean the matrix and concentrate BZF and PRX present in the samples collected from the laboratorial experiment as well as for the samples collected from the enriched slurry used in the bioaugmentation treatment. For the SPE extraction, cartridges Oasis HLB (3mL, 3cc) from Waters Corporation (Millford, MA, USA) were used in the ManiFold vacuum system (Supelco, Spain). The cartridges were conditioned with 5 mL of methanol, followed by 5 mL of deionized water. The samples were loaded and, afterwards, the cleaning step was performed using 5 mL of H₂O/MeOH (95:5 v/v) solution. The cartridges were then left to dry in vacuum for 30 minutes. The elution was carried out with 5 mL of MeOH/Formic Acid (96:4 v/v) solution which was evaporated by a flux of N₂ in bath at a temperature of approximately 30°C and the residue was dissolved in 0.5 mL of MeOH/H₂O (70:30 v/v) solution.

3.3.5 Sediments ultrasound extraction

For the determination of pharmaceuticals in the sediment samples, two sequential extractions were carried out with a solution of methanol/ammonia (95:5 v/v) using 5 mL per extraction in an ultrasonic bath (Transsonic 460/H). The supernatants were combined and evaporated until dryness under a N₂ flux. The residue was then dissolved in MeOH/H₂O (70:30 v/v) solution.

3.3.6 Pharmaceuticals compounds analysis

Sediment extracts and SPE solutions extracts were analysed by HPLC, in a VWR/HITACHI equipment (HPLC-DAD Elite LaChrom) with a diode array detector (DAD). The column was a 150 mm x 4.6 mm C18 Kinetex column (Phenomenex, UK). Two mobile phases (water/formic acid, 99:1 (v/v), and acetonitrile) were used, both always degassed for 15 min in the ultrasound.

The method consists of a 6 min gradient starting at 100 % of eluent A (H₂O/formic acid, 99:1 v/v) to 100 % of eluent B (acetonitrile). Then, gradient to 100 % of eluent A were reached again in 2 min, with a re-equilibration time of 2 min to restore the column. Flow rate gradient was maintained at 1 mL min^{-1} .

The sample injection volume was set at 50 μL and the detector signal was monitored at $\lambda = 252 \text{ nm}$ for BZF and $\lambda = 298 \text{ nm}$ for PRX. The limit of quantification was 0.1 mg L^{-1} and 0.2 mg L^{-1} for BZF and PRX, respectively.

3.4 Metal content determinations

Aliquots of each specific tissue of the plant (ca. 0.50 g), sediments and rhizosediments (ca. 0.25 g) were digested in closed PTFE vessels at high-pressure, with suitable amounts of concentrated HNO_3 65% (Merck) (1 mL for the different plant tissues and 5 mL for the sediments and rhizosediments), and 5 mL 30% H_2O_2 (Merck), only for plant tissues, using a microwave system (ETHOS 1, Milestone). The HNO_3 digestion of the sediments and rhizosediments only permits determination of total-recoverable metal contents, since it does not provide a complete dissolution of the sample, particularly silicates.

Total metal content in the different plant tissues and total-recoverable metal content in sediments and rhizosediments were determined by atomic absorption spectrophotometry either with flame atomization (AAnalyst 200, Perkin-Elmer), used for Cu, Fe, Mn, and Zn, or with electrothermal atomization (PinAAcle 900Z, Perkin-Elmer coupled to an AS 900 autosampler), used for Cd, Pb, Ni and Cr. Aqueous matched standards were used for external calibrations. Results were normalized by the samples dry weight and were expressed as $\mu\text{g g}^{-1}$.

3.5 Organic matter determination

The determination of organic matter in sediments followed the loss of ignition method. Vegetated and non-vegetated sediments were placed to dry at 105°C for 24 hours and then 2 g of sediment were weighted in ceramic cups. Triplicates were performed for each sediment sample. The weigh of the empty ceramic cups was also noted. Ceramic cups were placed in a muffle furnace (Carbolite type 301) at 500°C for 4 hours. After the cups were cooled down, they were weighted again, and the organic matter (OM) content was calculated according to the following equation:

$$OM (\%) = \left[1 - \frac{Cup + sediment_{before\ furnace} - Cup_{empty}}{Cup + sediments_{after\ furnace}} \right] \times 100$$

3.6 Statistical Analysis

Each sediment samples and plant tissue from the seasonal sampling were analysed in triplicate, being the mean and standard deviation calculated afterwards.

From the experiments, sediment sample from each vase was analysed individually and then the mean of the triplicates of each treatment was calculated.

For metals and organic matter levels significant ($p < 0.05$) differences among samples were evaluated through a parametric one-way analysis of variance (ANOVA).

4 Metals and pharmaceutical levels monitoring in Lima River estuary

4.1 Non-vegetated sediments

Metals' concentrations in non-vegetated sediments (Table 1) varied between 0.22 and 76 $\mu\text{g g}^{-1}$ for Cu, Zn, Mn, Pb, Cr, Ni, and Cd, and between 10.9 and 20 mg g^{-1} for Fe. Significant differences between the concentrations of Zn, Mn, Fe, Ni and Cr in non-vegetated sediments of the three locations were observed ($p < 0.05$). The highest levels were observed at site Sr^a das Areias. Different sources of contamination can influence the levels registered at the different sites.

The metals' levels in the analysed sediments allows to classify them as clean, according to Portuguese legislation (Caeiro, Costa et al. 2005), since the maximum values registered are all under the limit. Long *et al* defined a set of values to classify sediments as potentially toxic to organisms: effect range-low (ERL) and effect range-medium (ERM) (Long, Macdonald et al. 1995). At all sites, the levels observed were lower than their ERL.

Table 4.1 - Concentrations of Cu, Zn, Mn, Pb, Cr, Ni, Cd (in $\mu\text{g g}^{-1}$) and Fe (mg g^{-1}) observed in non-vegetated sediments (mean $\pm$ standard deviation, $n=3$). (C- Canoagem; S- Salinas; SA- Sr^a das Areias)

		Cu	Zn	Mn	Fe	Pb	Cr	Ni	Cd
First Sampling	L1	14.5 $\pm$ 0.7	67 $\pm$ 2	52 $\pm$ 3	18.6 $\pm$ 0.7	10 $\pm$ 0.2	18 $\pm$ 1	10 $\pm$ 2	<0.2 (LOD)
	L2.1	16 $\pm$ 3	72 $\pm$ 10	50 $\pm$ 6	20 $\pm$ 2	12.1 $\pm$ 0.8	16 $\pm$ 2	11 $\pm$ 1	<0.2 (LOD)
	L2.2	10 $\pm$ 1	51 $\pm$ 2	45 $\pm$ 4	14.6 $\pm$ 0.7	6.9 $\pm$ 0.6	12.0 $\pm$ 0.6	9 $\pm$ 2	<0.2 (LOD)
	L3	12.6 $\pm$ 0.9	57 $\pm$ 3	47 $\pm$ 3	17 $\pm$ 2	10.8 $\pm$ 0.2	14.3 $\pm$ 0.9	10 $\pm$ 2	<0.2 (LOD)
Second sampling	C	6.9 $\pm$ 0.8	33 $\pm$ 1	33.1 $\pm$ 0.8	10.9 $\pm$ 0.6	8 $\pm$ 3	10 $\pm$ 1	<2 (LOD)	<0.2 (LOD)
	S	10.2 $\pm$ 0.8	36 $\pm$ 4	28 $\pm$ 2	12 $\pm$ 1	11 $\pm$ 2	13 $\pm$ 2	<2 (LOD)	<0.2 (LOD)
	SA	10 $\pm$ 1	60 $\pm$ 8	55 $\pm$ 5	18 $\pm$ 1	14 $\pm$ 4	19 $\pm$ 2	4.7 $\pm$ 0.6	<0.2 (LOD)

Third sampling	C	5.1 ± 0.5	47 ± 2	33 ± 2	12.1 ± 0.6	15 ± 5	15.3 ± 0.6	<2 (LOD)	<0.2 (LOD)
	S	13 ± 1	75 ± 2	33.8 ± 0.6	15.8 ± 0.3	21 ± 6	25 ± 2	3 ± 1	0.22 ± 0.02
	SA	13.72 ± 0.02	76 ± 2	56 ± 1	19 ± 1	24 ± 7	23.6 ± 0.6	4.6 ± 0.9	0.19 ± 0.02

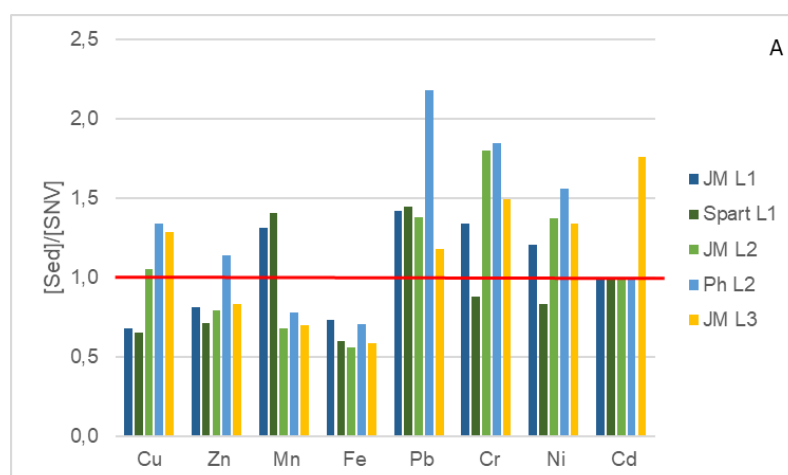
4.2 Vegetated versus non-vegetated sediments

The ratio between the metals' levels in vegetated and non-vegetated sediments was calculated and is presented in Figure 4.1 (A to C) for the first, second and third sampling campaigns, respectively. Metals levels in rhizosediments are presented in Appendix 1.

As it is possible to observe in Figure 4.1 A, for the first sampling campaign, the concentrations of Pb, Cr and Ni were higher in vegetated sediments in the sites L1, L2 and L3 (except the rhizosediments of *S. maritima* in site L2 for Cr and Ni). The sediments vegetated by *J. maritimus* (L2 and L3) and *P. australis* (L2) also registered higher levels of Cu than non-vegetated sediments in the same location. Both plants collected in site L1 registered higher levels of Mn in their rhizosediments than in the non-vegetated sediments.

Analysing Figures 4.1 B and C, it is clear that almost all the ratios between vegetated and non-vegetated sediments for sites Canoagem and Salinas are higher than 1, meaning that vegetated sediments have a higher metal concentration, contrary to the values registered for Sr^a das Areias. This is a clear indication that the location of Sr^a das Areias is very depleted compared to the other locations.

With the exception of Sr^a das Areias, this data clearly shows that all the plants collected, in both samplings, showed potential to retain metals in its rhizosediments, as it was previously observed in other studies (Almeida, Mucha et al. 2011).



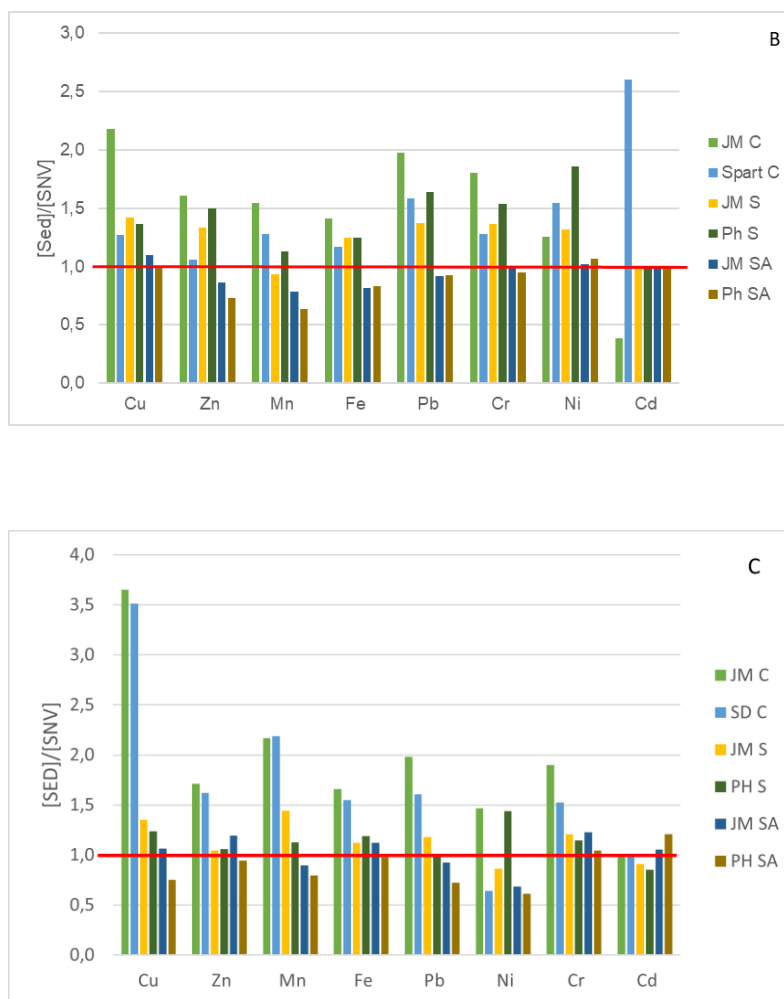


Figure 4.1 - Ratio between metals' levels in vegetated (Sed) and non-vegetated sediments (SNV): A- first sampling; B-second sampling; C-third sampling. (L1 and C colonized with *J. maritimus* and *S. maritima*; L2, S and SA colonized with *J. maritimus* and *P. australis*; L3 colonized with *J. maritimus*)

4.3 Metal in plant tissues

To determine if plants can uptake the metals or just retain them in their sediments, the ratio between the metals' concentration in the roots and non-vegetated sediments was calculated, as presented in Figure 4.2 (A to C). Figure 4.2 A clearly shows that the plants collected in site L2 have higher levels of Pb in their roots, compared to non-vegetated sediments, as the ratio is equal to 1.5. Thus, besides being able to retain this element in their sediments, *J. maritimus* and *P. australis* can uptake it and incorporate it in their roots.

Figure 4.2 B shows the results for the second sampling and, contrary to the vegetated sediments, the roots of the plants analysed can only retain Cu, in sites C and S as well. Copper is an element required by plants in small quantities for normal growth and development, therefore its uptake by plants is common (Sağlam, Yetişsin et al. 2016).

Regarding the third sampling, besides Cu, also Zn, Pb, Ni and Cd were retained by the roots of the plants. Once again, this was more evident in locations Canoagem and Salinas, contrasting clearly to what happens in location Sr^a das Areias.

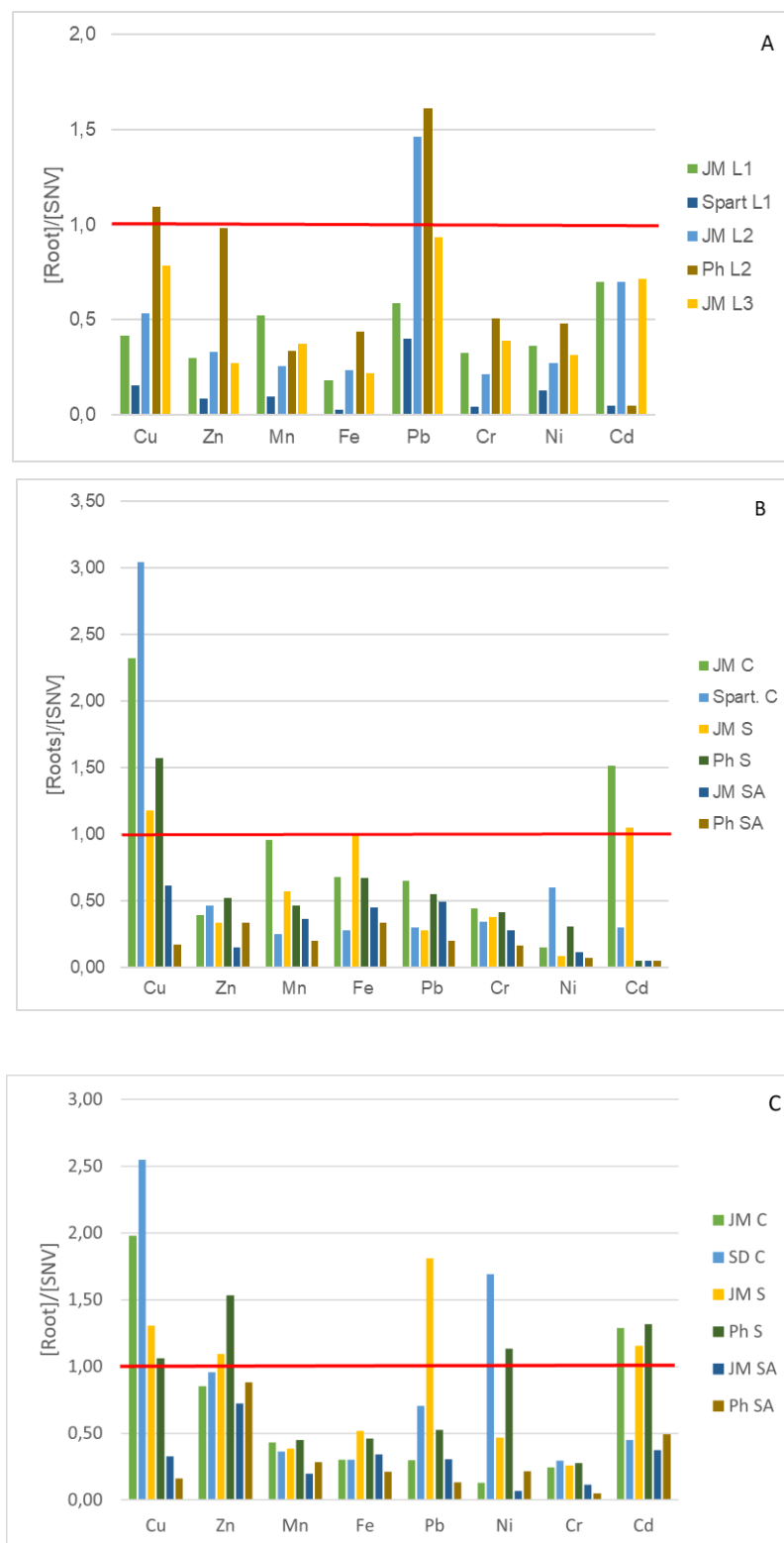


Figure 4.2 - Ratio between metals' levels in roots (Root) and non-vegetated sediments (SNV): A-first sampling; B-second sampling; C-third sampling. (L1 and C colonized with *J. maritimus* and *S. maritima*; L2, S and SA colonized with *J. maritimus* and *P. australis*; L3 colonized with *J. maritimus*)

To understand if the roots are the only plant part capable of retaining metals, its levels in rhizomes (when present) and aerial part were determined. Metal levels in plant tissues (roots, rhizomes and aerial part) are shown in Appendix 1. It was possible to observe that the highest concentrations are mainly registered in the roots of the three plant species, which indicates that, in the cases where the plants uptake metals by their roots, they are not incorporated in other plant parts. The plants' capacity to accumulate certain metals depends on its uptake capacity and intracellular binding sites. The mobilization of the metals and their uptake from the soil, as well as the compartmentation and sequestration within the root, are key processes that influence metal accumulation in plants (Clemens, Palmgren et al. 2002).

The levels of organic matter in rhizosediments were also assessed to understand the plants' capacity to retain metals. High levels of organic matter are usually present in rhizosediments with high metal concentrations since the material exudated by the plants' roots can form complexes with metals, imprisoning them in these areas (Almeida, Mucha et al. 2011). The results obtained are presented in Table 4.2. In this case, there is a significant difference between the organic matter levels in the three locations ($p < 0.05$), as locations Canoagem and Salinas had higher levels of organic matter in its sediments, compared to location Sr^a das Areias, which is according to the results obtained previously. Besides, organic matter was in general higher in vegetated sediment than in non-vegetated one.

Table 4.2 - Organic matter content of the collected sediments in the three locations (mean $\pm$ standard deviation, $n=3$). (SNV-non vegetated sediment; JM-rhizosediment of *J. maritimus*; SM-rhizosediment of *S. maritima*; PH-rhizosediment of *P. australis*)

	Canoagem			Salinas			S ^a das Areias		
	SNV	JM	SM	SNV	JM	PH	SNV	JM	PH
Second sampling	2.91 $\pm$ 0.07	8.3 $\pm$ 0.2	11.1 $\pm$ 0.4	7.5 $\pm$ 0.2	12.3 $\pm$ 0.4	10.4 $\pm$ 0.2	5.1 $\pm$ 0.1	7.6 $\pm$ 0.4	8.3 $\pm$ 0.3
Third sampling	3.3 $\pm$ 0.1	11.7 $\pm$ 0.2	12.1 $\pm$ 0.7	6.3 $\pm$ 0.1	13.3 $\pm$ 0.1	10.98 $\pm$ 0.06	7.2 $\pm$ 0.1	9.2 $\pm$ 0.2	5.8 $\pm$ 0.1

Comparing with previous work performed in the Lima River (Almeida, Mucha et al. 2011), the concentration of the several metals assessed in this project were lower than the levels detected in 2010.

4.4 Pharmaceuticals in sediments

Sediments were extracted and the supernatants were analysed through HPLC. An example of the obtained chromatogram is represented in Figure 4.3. Comparing the blue line, which represents a sediment sample, and the green line, a 4 mg L⁻¹ standard solution of the respective pharmaceutical prepared in the same day as the analysis, it is possible to observe that no pharmaceutical was detected in the sample, either for BZF or PRX (top and bottom image, respectively). This was observed for all the analysed sediments, representing all the locations where vegetated and non-vegetated sediments samples were collected.

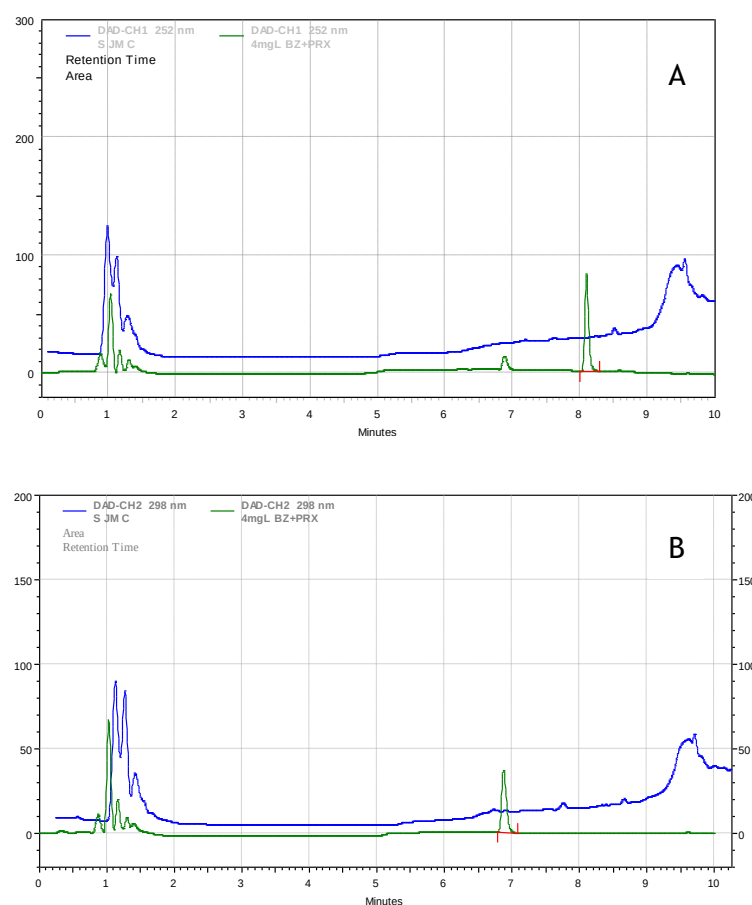


Figure 4.3 - Example of chromatograms obtained by HPLC for sediments analysed (A: BZF chromatogram; B: PRX chromatogram; Blue line: sediment sample; Green line: standard)

5 Pharmaceuticals removal in the microcosms and estuarine replication systems

5.1 Microcosms pharmaceuticals removal

For monitoring the pharmaceuticals concentration in the microbial consortium medium at the end of each two-weeks cycle, an aliquot of medium from each microcosm was collected and analysed to determine the pharmaceuticals concentration.

Regarding the microcosms doped with BZF, at the end of the first cycle BZF was detected but at values lower than the limit of quantification (0.1 mg L^{-1}), whereas at the end of the second cycle, no BZF was detected in the culture medium. In the microcosms doped with PRX, the compound was not detected in the medium since the first cycle. So, considering the limit of quantification of 0.1 mg L^{-1} , a removal of $>90 \%$ is assumed for both pharmaceuticals.

This removal may be due to degradation by native estuarine organisms present in the sediment or retention of the compounds in the sediments, as previously reported in other works (Duarte, Almeida et al. 2019, Dias, Correia et al. 2020). Abiotic factors such as photodegradation could contribute to the degradation of BZF and PRX (Trovó, Melo et al. 2008), however this was not expected to influence the pharmaceuticals concentration since the flasks were kept in the dark during the whole experiment. Adsorption to sediment and/or biomass can be another abiotic factor that can contribute for removal of these pharmaceuticals, a feature previously observed for PRX but not for BZF (Duarte et al., 2019).

5.2 Bioaugmentation coupled with *P. australis* bioremediation potential

At the end of the first cycle, it was added to each vase $140 \text{ }\mu\text{g}$ of the respective pharmaceutical (7 days of watering in which 200 mL with a concentration of $100 \text{ }\mu\text{g L}^{-1}$ was added each day). During this period, water was collected from the vases in the first two watering days, no water coming out of the systems in the following days. The mass of each compound present in the collected water was calculated and is presented in Table 4.3. Only BZF was detected in the water collected from the systems doped with BZF, accounting for less than 2% of the total amount added. No PRX was detected in the collected water.

Table 5.1 - Volume and mass of BZF collected from the vases and percentage relatively to total amount added. (CT B represents the vases for control doped with BZF, BB represents the vases for bioaugmentation with BZF enriched slurry)

	Day 2 after doping		Day 4 after doping		m _{total}	%
	Volume (mL)	Mass (µg)	Volume (mL)	Mass (µg)		
CT B1	0	0	10	0.357	0.357	0.3
CT B2	160	0.269	90	0.887	1.156	0.8
CT B3	90	1.284	80	1.506	2.790	2.0
BB1	5	<LOD	40	0.931	0.931	0.7
BB2	75	0.569	10	0.410	0.979	0.7
BB3	90	0.644	40	0.487	1.131	0.8

Regarding the sediment samples collected at the end of the cycle, for the systems doped with BZF, similar peaks appeared in all 6 chromatograms, one minute later than the usual retention time of this compound. To confirm if the peaks corresponded to the compound, two samples were reanalysed and immediately doped with BZF. As it is possible to observe in Figure 4.4, the peak around minute 9 remained the same, and a small peak appeared at the usual retention time of BZF, which confirms that the peak did not correspond to the pharmaceutical. This peak could correspond to a transformation product of BZF, however the analysis of the sample through other techniques such as mass spectroscopy would be necessary to confirm this hypothesis. BZF, if present, was not detected in any of the sediment samples.

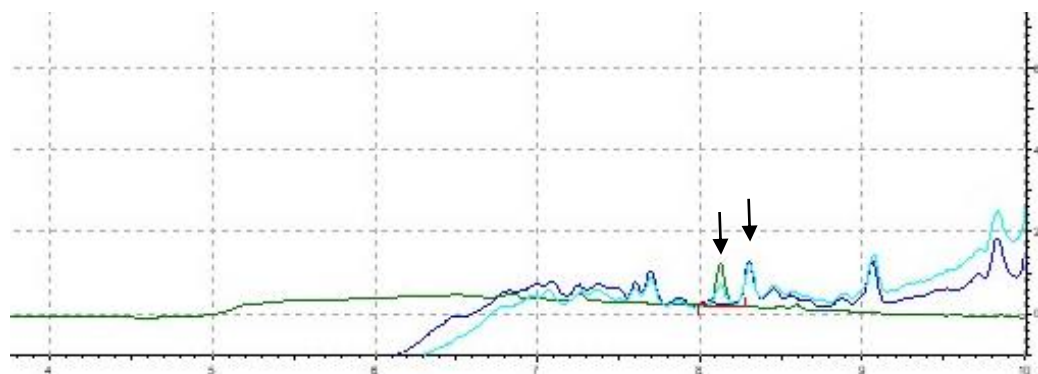


Figure 5.1 - Chromatogram of sediment sample BB2. (Dark blue line: sediment sample; Light blue line: sediment sample doped with BZF; Green line: standard)

The matrix effect was also analysed by calculating its percentage comparing the peaks' area of the doped sediment and of a standard with the same concentration, according to the post extraction addition protocol (Zhou, Yang et al. 2017). The matrix effect obtained was 9 %, which indicates a slight signal suppression. To get more reliable results, the calibration curve from a matrix matched standard solutions should be compared with a calibration curve of standard solutions, applying a matrix effect factor to the obtained results.

As for PRX, this compound was detected in two sediment samples (two vases) from the control group, however only for one sample the values were above the limit of quantification, resulting in a concentration of $0.19 \mu\text{g g}^{-1}$. In the vases where slurry was added as bioaugmentation, PRX was detected only in one of the samples collected (one out of three vases), resulting in a concentration of $0.23 \mu\text{g g}^{-1}$. Considering that a total amount of $140 \mu\text{g}$ of PRX was added to each vase during the entire cycle (two weeks), it is possible to assume that the retention of the compounds in the sediments played a big role, although the amount of PRX when detect was very low.

Considering the compounds concentration in water and sediment samples, it could be assumed that both pharmaceuticals were removed by the joint action of the microorganisms and plants present in the vases. The compounds may have been degraded by the mentioned agents, resulting in transformation products that cannot be detected/identified with the techniques used in this work. The uptake of organic compounds such as pharmaceuticals by *P. australis* has been reported as dependent on the $\log K_{OW}$ of the substance; if this value is between 1 and 3, meaning that the compound has a moderate hydrophobicity, the plant has highest uptake capabilities (Sauvêtre and Schröder 2015). PRX has a $\log K_{OW}$ value of 1.23 at neutral pH so probably plant uptake could explain part of the removal that occurred. The same is not likely to be true for BZF since its $\log K_{OW}$ is above 3, meaning that it is more likely to adsorb to sediment organic matter such as the plants roots or indeed suffer other biodegradation processes.

Due to the high natural attenuation efficiency of the systems, no differences were observed between the control groups and the ones where microbial consortia was added as bioaugmentation, proving the high bioremediation potential of *P. australis* and its associated microbial community in a simulated estuarine environment. As it was previously reported, *P. australis*, either by itself or in the presence of its sediment microbial communities, was able to remove BZF and PRX from a simplified estuarine medium (Dias, Correia et al. 2020). Other studies highlighted that the presence of salt marsh plants, in this case *S. maritima* and *H. portulacoides*, can enhance the remediation of pharmaceuticals and personal care products from estuarine environment (Couto, Ferreira et al. 2018). This data allows to understand the relevance of salt marshes in contaminated estuarine locations as they have the potential to

retain not only ECs but also metals (Almeida, Mucha et al. 2011), contributing to the regeneration of the ecosystem.

6 Conclusion and Assessment of the work done

The concentration of several metals, as well as the presence of BZF and PRX, in estuarine sediments, non-vegetated and vegetated with *Phragmites australis*, *Juncus maritimus* and *Spartina maritima* was assessed. The metals levels in the sediments were all under the legal limit which allows to classify them as clean. This monitoring is relevant since the presence of several metals may interfere with the plants ability to retain certain compounds. Neither in the rhizosediments or non-vegetated sediments was detected the target pharmaceuticals.

Following this work, an experiment was assembled to evaluate the bioremediation potential of *P. australis* and its associated microbial community in a simulated estuarine environment contaminated with BZF and PRX. Moreover, a slurry obtained from the rhizosediment of the mentioned plant was used to obtain an enriched slurry resistant to both pharmaceutical to understand if the bioaugmentation with this slurry would improve the systems' capacity to remove the compounds. Regarding the obtained results, it was possible to observe that the enriched slurry itself had the potential to remove BZF and PRX, individually, from the medium, either by adsorption to the sediments/biomass or biodegradation by the native microorganisms. Total removal was obtained by the end of the second cycle. Regarding the laboratorial experiment simulating estuarine environment, the bioaugmentation treatment did not enhance the systems removal potential since both groups showed potential for the complete removal of both compounds (natural attenuation).

The end destination of the compounds requires further research since many mechanisms can lead to their removal. Plants can uptake them through the roots and into their structures or in association with its native microorganisms degrade the compounds to other less toxic forms. The compounds can also be retained in rhizosediments. From this study, it is possible to conclude that *P. australis*, its associated microorganisms and the rhizosediments that surround it have the potential to remove BZF and PRX from a contaminated estuarine environment, with and without a bioaugmentation treatment to help the process.

The two main objectives of this work were to monitor the presence of several metals and two pharmaceuticals, BZF and PRX, in non-vegetated sediments and sediments vegetated by three salt marsh plants in the Lima River, and to assess the bioremediation potential of *P. australis* and its native microbial community for the removal of emerging contaminants from an estuarine environment. A bioaugmentation treatment was also designed to understand if it would improve this bioremediation potential. Both objectives were successfully accomplished.

The experiment is expected to carry on with at least 4 more cycles so the pharmaceutical concentration levels in the sediments can be monitored and the efficiency of the bioaugmentation treatment evaluated.

Although the estuarine environment was simulated as best as possible, the conditions present in the study are still simplified compared to what occurs in estuaries, such as tides and continuous input of contaminants. Other limitation was the equipment available to perform the analysis, as the use of mass spectroscopy would allow better understanding of some of the obtained results.

In future work, additional contaminants can be added to the systems, like a metal, to determine its influence in the plants bioremediation potential as the presence of multiple contaminants can alter the plants phytoremediation potential (Fraga-Santiago, Dias et al. 2022). It would also represent more accurately what occurs in an estuary where multiple contaminants are present.

It would also be relevant to understand the changes in the structure of the microbial community present in the systems along the experiment. During the course of this work, it was not possible to perform this analysis, however samples are collected and stored for a future work which will help to understand a few of the processes mentioned in this study. Due to removal potential observed for the enriched slurry obtained from the *P. australis* rhizosediments, it is planned to stimulate this culture to a point where the isolation and cultivation of the culturable bacterial community is possible. Afterwards, these microorganisms can be cryopreserved and used in other experiments to further assess their degradation ability.

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Appendix 1 Metals concentration in plant parts and rhizosediments

Table A1 1 - Concentrations of Cu, Zn, Mn, Pb, Cr, Ni, Cd ($\mu\text{g g}^{-1}$) and Fe (mg g^{-1}) observed in rhizosediments (mean $\pm$ standard deviation, $n=3$). (C- Canoagem; S- Salinas; SA- Sr^a das Areias)

		Cu	Zn	Mn	Fe	Pb	Cr	Ni	Cd
First Sampling	JM L1	9.9 $\pm$ 0.2	55 $\pm$ 2	68 $\pm$ 2	13.6 $\pm$ 0.7	14 $\pm$ 1	23 $\pm$ 2	12 $\pm$ 1	<0.2
	JM L2	17 $\pm$ 1	57 $\pm$ 1	33.9 $\pm$ 0.2	11.3 $\pm$ 0.4	16.6 $\pm$ 0.5	29 $\pm$ 1	15 $\pm$ 2	<0.2
	JM L3	16.2 $\pm$ 0.8	47.7 $\pm$ 0.7	32 $\pm$ 1	10.0 $\pm$ 0.5	12.7 $\pm$ 0.8	21 $\pm$ 2	13.8 $\pm$ 0.3	0.352 $\pm$ 0.004
	JM L4	10 $\pm$ 2	34 $\pm$ 1	27.6 $\pm$ 0.3	12 $\pm$ 1	25.8 $\pm$ 0.9	12.4 $\pm$ 0.3	7.1 $\pm$ 0.8	<0.2
	SM L1	9.4 $\pm$ 0.2	48 $\pm$ 3	72 $\pm$ 2	11.1 $\pm$ 0.3	14.4 $\pm$ 0.8	15.3 $\pm$ 0.4	8.6 $\pm$ 0.5	<0.2
	PH L2	12.7 $\pm$ 0.3	58 $\pm$ 1	35.3 $\pm$ 0.2	10.3 $\pm$ 0.4	15.0 $\pm$ 0.5	22 $\pm$ 3	14.2 $\pm$ 0.2	<0.2
Second sampling	JM C	15 $\pm$ 1	53 $\pm$ 4	51 $\pm$ 2	15.4 $\pm$ 0.3	15.8 $\pm$ 0.5	18 $\pm$ 1	4.4 $\pm$ 0.5	<0.2
	SM S	19 $\pm$ 1	56 $\pm$ 6	65 $\pm$ 5	18 $\pm$ 2	25 $\pm$ 2	23 $\pm$ 2	6.8 $\pm$ 0.6	<0.2
	JM S	14.5 $\pm$ 0.6	48 $\pm$ 7	26.2 $\pm$ 0.4	15 $\pm$ 2	15.1 $\pm$ 0.3	17.7 $\pm$ 0.6	4.6 $\pm$ 0.4	<0.2
	PH S	13.9 $\pm$ 0.4	54 $\pm$ 3	31.7 $\pm$ 0.9	15 $\pm$ 1	18 $\pm$ 5	20 $\pm$ 1	6.5 $\pm$ 0.3	<0.2
	JM SA	11 $\pm$ 1	52 $\pm$ 4	43.1 $\pm$ 0.4	14.7 $\pm$ 0.7	12.8 $\pm$ 0.7	19 $\pm$ 2	4.8 $\pm$ 0.5	<0.2
	PH SA	10 $\pm$ 2	44 $\pm$ 1	35 $\pm$ 4	15 $\pm$ 3	13 $\pm$ 1	18 $\pm$ 3	5 $\pm$ 2	<0.2
d sam	JM C	18.6 $\pm$ 0.5	81 $\pm$ 2	72 $\pm$ 3	20.2 $\pm$ 0.8	29.7 $\pm$ 0.3	29 $\pm$ 2	1.9 $\pm$ 0.9	<0.2

	SM C	18 ± 1	76 ± 1	72 ± 4	18.9 ± 0.6	24 ± 2	23.4 ± 0.4	0.8 ± 0.2	<0.2
	JM S	17.6 ± 0.4	78 ± 3	49 ± 4	18 ± 1	25 ± 3	30 ± 1	2.6 ± 0.4	<0.2
	PH S	16.1 ± 0.3	79 ± 1	38.1 ± 0.4	18.8 ± 0.4	21 ± 3	29 ± 2	4.3 ± 0.8	0.19 ± 0.04
	JM SA	15 ± 2	91 ± 4	50 ± 1	21 ± 2	22 ± 2	29.0 ± 0.8	3.2 ± 0.3	<0.2
	PH SA	10.3 ± 0.5	72 ± 3	44 ± 2	18.6 ± 0.5	17 ± 1	25 ± 1	2.8 ± 0.3	0.23 ± 0.07

Table A1 2 - Concentrations of Cu, Zn, Mn, Pb, Cr, Ni, Cd ($\mu\text{g g}^{-1}$) and Fe (mg g^{-1}) observed in roots (mean ± standard deviation, n=3). (C- Canoagem; S- Salinas; SA- Sr^a das Areias)

		Cu	Zn	Mn	Fe	Pb	Cr	Ni	Cd
First Sampling	JM L1	6.0 ± 0.6	20 ± 1	27 ± 3	3.41 ± 0.04	6 ± 1	5.6 ± 0.2	3.8 ± 0.8	0.14 ± 0.01
	JM L2	8.5 ± 0.9	23.8 ± 0.8	13 ± 2	4.7 ± 0.4	18 ± 2	3.5 ± 0.4	2.9 ± 0.7	0.14 ± 0.02
	JM L3	9.9 ± 0.8	16 ± 2	17 ± 2	3.8 ± 0.2	10 ± 4	6 ± 1	3.3 ± 0.7	0.143 ± 0.001
	JM L4	4.6 ± 0.4	11.9 ± 0.9	5 ± 1	3.2 ± 0.9	7 ± 2	1.0 ± 0.2	3 ± 1	0.11 ± 0.05
	SM L1	2.2 ± 0.1	5.8 ± 0.9	5.0 ± 0.1	0.5 ± 0.1	4.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	<0.01
	PH L2	10.4 ± 0.5	50.1 ± 0.8	15 ± 1	6 ± 1	11 ± 3	6 ± 1	4.4 ± 0.8	<0.01
Second sampling	JM C	16 ± 1	12.9 ± 0.6	32 ± 8	7.4 ± 0.6	5.2 ± 0.5	4.43 ± 0.03	0.52 ± 0.08	<0.01
	SM S	21 ± 3	15.2 ± 0.7	8 ± 2	3.0 ± 0.3	2.4 ± 0.3	3.4 ± 0.8	2.1 ± 0.8	0.06 ± 0.01
	JM S	12 ± 1	12 ± 1	16 ± 3	12 ± 1	3 ± 1	4.9 ± 0.1	0.3 ± 0.1	0.21 ± 0.01
	PH S	16.0 ± 0.7	18.6 ± 0.4	13 ± 2	8 ± 1	6 ± 1	5.3 ± 0.7	1.06 ± 0.09	<0.01

	JM SA	6.1 ± 0.5	9 ± 1	20 ± 1	8.1 ± 0.9	6.9 ± 0.5	5.2 ± 0.9	0.53 ± 0.03	<0.01
	PH SA	<1.6	20 ± 2	11 ± 4	6 ± 1	2.8 ± 0.3	3 ± 1	<0.33	<0.01
Third sampling	JM C	10.1 ± 0.7	40.1 ± 0.7	14.2 ± 0.8	3.7 ± 0.3	4.5 ± 0.5	3.7 ± 0.5	0.17 ± 0.05	0.258 ± 0.002
	SM C	13 ± 2	45 ± 3	12 ± 1	3.7 ± 0.4	10.6 ± 0.8	4.5 ± 0.2	2.2 ± 0.5	0.09 ± 0.02
	JM S	17 ± 2	82 ± 4	13 ± 2	8.2 ± 0.2	38 ± 4	6.5 ± 0.4	1.4 ± 0.3	0.254 ± 0.009
	PH S	13.1 ± 0.6	115 ± 12	15.2 ± 0.3	7.3 ± 0.5	11 ± 2	6.9 ± 0.2	3.4 ± 0.8	0.29 ± 0.01
	JM SA	4.5 ± 0.3	55 ± 5	11 ± 2	6.5 ± 0.5	7.3 ± 0.2	2.7 ± 0.1	0.32 ± 0.03	0.07 ± 0.01
	PH SA	2.2 ± 0.3	67 ± 10	16 ± 3	4 ± 1	3.2 ± 0.5	1.2 ± 0.4	1.0 ± 0.2	0.09 ± 0.01

Table A1 3 - Concentrations of Cu, Zn, Mn, Pb, Cr, Ni, Cd ($\mu\text{g g}^{-1}$) and Fe (mg g^{-1}) observed in rhizomes (mean ± standard deviation, n=3). (C- Canoagem; S- Salinas; SA- Sr^a das Areias)

		Cu	Zn	Mn	Fe	Pb	Cr	Ni	Cd
First Sampling	JM L1	9.8 ± 0.8	23 ± 6	4 ± 1	0.15 ± 0.03	0.7 ± 0.4	1.0 ± 0.5	9 ± 1	0.04 ± 0.01
	JM L2	6 ± 2	18 ± 7	6.0 ± 0.3	2.1 ± 0.6	1.2 ± 0.3	1.4 ± 0.3	6 ± 3	<0.02
	JM L3	9 ± 1	12 ± 9	6 ± 2	0.8 ± 0.2	0.9 ± 0.1	1.3 ± 0.6	4 ± 2	<0.02
	JM L4	13 ± 6	25 ± 8	2.9 ± 0.6	2.8 ± 0.1	3 ± 2	0.3 ± 0.1	1.4 ± 0.1	<0.02
Second sampling	JM C	5 ± 2	16 ± 2	13 ± 1	2.0 ± 0.2	0.45 ± 0.08	0.8 ± 0.2	<0.2	0.084 ± 0.005
	JM S	3.9 ± 0.7	14 ± 4	13 ± 6	0.7 ± 0.3	0.3 ± 0.09	0.4 ± 0.2	<0.2	0.01 ± 0.03
	JM SA	4.0 ± 0.5	18 ± 2	12 ± 2	1.8 ± 0.2	0.41 ± 0.04	1.2 ± 0.2	0.3 ± 0.1	0.027 ± 0.009

Third sampling	JM C	9 ± 2	79 ± 12	3.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.174 ± 0.003
	JM S	14 ± 1	41 ± 6	4 ± 1	1.3 ± 0.2	1.0 ± 0.2	0.4 ± 0.1	0.14 ± 0.03	0.11 ± 0.01
	JM SA	3.5 ± 0.4	15 ± 1	8 ± 2	1.8 ± 0.5	0.6 ± 0.3	0.7 ± 0.4	0.13 ± 0.07	0.036 ± 0.009

Table A1 4 - Concentrations of Cu, Zn, Mn, Pb, Cr, Ni, Cd ($\mu\text{g g}^{-1}$) and Fe (mg g^{-1}) observed in aerial plant part (mean ± standard deviation, n=3). (C- Canoagem; S- Salinas; SA- Sr^a das Areias)

		Cu	Zn	Mn	Fe	Pb	Cr	Ni	Cd
First Sampling	JM L1	2.5 ± 0.3	5.2 ± 0.6	11.4 ± 0.9	0.11 ± 0.04	0.16 ± 0.07	0.27 ± 0.01	<0.2	0.06 ± 0.02
	JM L2	1.5 ± 0.2	4.4 ± 0.7	17 ± 2	0.17 ± 0.01	0.40 ± 0.07	0.35 ± 0.06	<0.2	<0.02
	JM L3	2.7 ± 0.7	3 ± 1	19 ± 4	0.07 ± 0.02	0.15 ± 0.01	0.3 ± 0.14	<0.2	<0.02
	JM L4	2.1 ± 0.3	5 ± 1	13 ± 1	0.12 ± 0.04	0.19 ± 0.06	<0.1	<0.2	<0.02
	SM L1	<1	13 ± 3	2.0 ± 0.6	0.07 ± 0.02	0.13 ± 0.01	<0.1	<0.2	<0.02
	PH L2	<1	4.7 ± 0.2	1.7 ± 0.3	0.06 ± 0.01	0.15 ± 0.05	<0.1	<0.2	<0.02
Second sampling	JM C	5.0 ± 0.7	2.3 ± 0.2	13.2 ± 0.9	0.18 ± 0.07	0.13 ± 0.03	0.17 ± 0.07	<0.2	0.040 ± 0.001
	SM S	1.6 ± 0.2	5 ± 2	<1	0.38 ± 0.08	0.26 ± 0.07	0.9 ± 0.3	0.4 ± 0.1	0.015 ± 0.012
	JM S	3.0 ± 0.2	2.1 ± 0.6	13 ± 2	0.08 ± 0.03	<0.1	<0.1	<0.2	0.018 ± 0.002
	PH S	1.09 ± 0.02	3 ± 1	<1	0.10 ± 0.05	<0.1	<0.1	<0.2	<0.01
	JM SA	2.1 ± 0.3	1.8 ± 0.5	16 ± 1	0.20 ± 0.05	0.34 ± 0.05	0.2 ± 0.1	<0.2	<0.01
	PH SA	<1	7 ± 4	<1	0.13 ± 0.07	<0.1	<0.1	<0.2	<0.01

Third sampling	JM C	3.2 ± 0.1	12 ± 1	5.3 ± 0.2	0.13 ± 0.03	0.22 ± 0.04	0.17 ± 0.06	0.18 ± 0.02	0.060 ± 0.004
	SM C	1.54 ± 0.4	10 ± 1	5.0 ± 0.2	0.29 ± 0.03	0.6 ± 0.2	0.8 ± 0.3	0.6 ± 0.2	0.014 ± 0.002
	JM S	3.5 ± 0.1	8.8 ± 0.4	12.1 ± 0.7	0.06 ± 0.02	0.1 ± 0.1	0.10 ± 0.09	<0.1	0.041 ± 0.002
	PH S	1.0 ± 0.4	10 ± 3	1.6 ± 0.4	0.13 ± 0.07	0.26 ± 0.07	0.3 ± 0.1	<0.1	0.018 ± 0.006
	JM SA	2.0 ± 0.1	7.5 ± 0.2	32 ± 2	0.13 ± 0.01	0.31 ± 0.03	0.14 ± 0.01	<0.1	0.030 ± 0.004
	PH SA	1.2 ± 0.02	21 ± 1	9 ± 2	0.28 ± 0.04	0.5 ± 0.2	0.5 ± 0.2	0.26 ± 0.06	0.021 ± 0.008