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PHYTOREMEDIATION OF PHARMACEUTICALS BY ESTUARINE SALT MARSH PLANTS

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"*Leave this world a little better than you found it.*"

Robert Baden-Powell

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Abstract

Continued worldwide industrialization and exponential growth of human population has led to an increased release of several types of pollutants to the environment, causing extensive environmental and human health problems. These pollutants include those known for some time to be of environmentally dangerous and those which are only recently having attention from environmental regulators, for instance emerging contaminants (ECs), such as human and veterinary pharmaceuticals, personal care products, steroid hormones, among others, being most of them not regulated.

Estuaries are one of the most affected environments with the release of these contaminants, being considered sinks for contaminants, therefore it is imperative new remediation and recovery strategies for these important ecological areas.

This study aimed to, firstly, carry out a survey of the presence of two pharmaceuticals (paroxetine and bezafibrate) in different estuarine sediments (vegetated and non-vegetated) from two estuaries in the North of Portugal (two samples from Lima river estuary and one sample from Cávado river estuary). In second place, controlled laboratory experiments were carried out to evaluate the potential of a salt marsh plant (*Phragmites australis*) and its rhizospheric microorganisms to degrade the selected pharmaceuticals, including in the presence of another estuarine contaminant (copper).

Considering the first survey, bezafibrate was detected in the rhizosediments of two of the three samples analyzed and paroxetine was detected in the three rhizosediments analyzed. But none of the compounds was detected in non-vegetated sediments, indicating the plant had a role in the distribution of these compounds in estuarine areas.

Regarding the laboratory experiments, in the systems without copper, the plant and its rhizosediment, when separated, showed a removal of 42% and 45% for bezafibrate and of 57% and 82% for paroxetine. When combined, a removal efficiency of 51% for bezafibrate and 90% for paroxetine was observed. In the systems with copper, the plant and its rhizosediment, when separated, showed a removal of 43% and 46% for bezafibrate and of 70% and 89% for paroxetine and when combined, presented a removal efficiency of 75% for bezafibrate and 95% for paroxetine, which demonstrates that no substantial differences were detected by the presence of copper.

Overall, the plant and specially it rhizosediments and the microorganisms associated have potential to remove these contaminants from estuarine environment and eventually degrade the selected pharmaceutical compounds, a feature that requires more research.

Keywords: phytoremediation, pharmaceuticals, estuary, salt-marsh plant

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Notation and Glossary

CWs – Constructed wetlands

DAD – diode array detector

ECs – emerging contaminants

El – Elutriate treatment

El + Pl – Elutriate and plant treatment

El + Sed - Elutriate and rhizosediment treatment

El+Pl+Sed - Elutriate, plant and rhizosediment treatment

GC – Gas chromatography

HPLC – High Performance Liquid Chromatography

K_{OW} - Octanol-water partition coefficient

LC – Liquid chromatography

LOD – Limit of detection

LOEC – Lowest observed effect concentration

MS – Mass spectrometer

PAHs – Polycyclic aromatic hydrocarbons

PPCPs – Pharmaceuticals and personal-care products

SPE – Solid-phase extraction

SSRI – Selective serotonin uptake inhibitor

WWTP – Wastewater treatment plant

1 Introduction

1.1 Framing and presentation of the work

An increase in the demand for the Earth's limited supply of freshwater has been originated by the exponential growth of human population. This exponential growth also leads to higher input of contaminants into the environment, including contaminants which are only recently getting the attention from environmental regulators, i.e. emerging contaminants (ECs). ECs are a big concern because they are natural or synthetic substances that when present in the environment can cause damaging effects at ecological or human level, being most of them not regulated. These contaminants include, among others, human and veterinary pharmaceuticals, personal care products, steroid hormones and surfactant wastes (Petrović et al. 2003).

In this work, it will be given a special attention to pharmaceuticals. Pharmaceuticals are synthetic or natural chemicals used in medicines and are widely and gradually being utilized as a part of human and veterinary medication. The production and consumption of pharmaceuticals are increasing across both developed and developing countries, not only in terms of accessibility, but also the volume or quantity of drugs consumed (Marsik et al. 2017) and Portugal follows this increase, which leads to higher levels of pharmaceuticals released into the environment.

Pharmaceuticals have been found in Portuguese rivers and estuaries as in other parts of the world, mainly due to inefficient removal at wastewater treatment plants (WWTPs), being released every day to surface water in WWTPs effluents.

Estuaries are highly productive ecosystems that have an important place in biogeochemical cycles, however they are also very fragile ecosystems that suffer from high anthropogenic pressure receiving all type of contaminants (Fernandes et al. 2017a). Most of these contaminants can be dissolved in water, accumulated in estuarine sediments and/or bioaccumulated in organisms (Sun et al. 2012), causing serious effects in several organisms, ecosystem degradation, habitats deterioration and possible human poisoning, for this reason, estuaries are considered sinks for contaminants, being imperative new remediation and recovery strategies for these important ecological areas (Fernandes et al. 2017a).

A possible method to recover and remediate contaminated environments is phytoremediation. Phytoremediation offers a good alternative to the traditional methods. This technology, based on the natural processes, uses plants and associated microorganisms to remove, accumulate, metabolize, absorb and/or degrade organic and inorganic pollutants from contaminated sites (soil, water and air) (Fernandes et al. 2017a). It is a cost-effective, promising and trustworthy

technology and represents a sustainable solution to recover damaged ecosystems, such as estuarine areas (Feng et al. 2017; Fernandes et al. 2017b).

This study aims to explore the application of phytoremediation for the removal of pharmaceuticals from estuarine areas, using autochthonous salt marsh plant.

1.2 Organization of the thesis

This thesis is organized in six main chapters. In the present chapter (1), the Introduction can be found, in which are included the framing and presentation of the work and its organization.

Chapter 2 concerns the Context and State of the art, in which a contextualization about the problem associated with this study, framing it in a global context and describing the different topics covered in the thesis, such as emerging contaminants, phytoremediation, estuaries and salt-marshes, pharmaceuticals analyzed, technologies used, among others.

Materials and methods used in the development of the laboratory experiments, samples extraction and analysis are presented in chapter 3.

In chapter 4 results of the initial survey and the laboratory experiment are presented and discussed for each pharmaceutical analyzed.

The final conclusions taken after this thesis are resumed in chapter 5.

Lastly, in chapter 6 a final assessment about this study is done, identifying the achieved objectives, the other work carried out, the limitations and future work.

2 Context and State of the art

The exponential increase of human population has originated a higher demand for the Earth's limited supply of freshwater. Therefore, preserving water resources is one of the biggest environmental issues of the 21st century.

Contamination of aquatic environments by every type of pollutant remains a challenging issue mainly when it affects freshwaters that are a source of food, drinking water, and recreation (Kim and Aga 2007).

In the last years, an increasing concern with the potential adverse effects to both human and aquatic organisms resulting from the production, use and disposal of chemicals in industry, agriculture, medical treatment, and even common household conveniences (Kolpin et al. 2002).

Additionally, higher population density also leads to higher input of current industrial chemical contaminants including those that have long been present in the environment like metals and polycyclic aromatic hydrocarbons (PAHs), and those which are only recently having attention from environmental regulators, such as emerging contaminants (ECs).

2.1 Emerging contaminants

Emerging contaminants (ECs) are natural or synthetic substances that when present in the environment can cause damaging effects at ecological or human level, being most of them not regulated (Figure 2.1).

In other words, ECs are any substances that are beginning to be suspected to cause harm, and they can be new substances, or they may have been around for a long time but only recently have been detected in the environment. Studies regarding ECs effects on the environment or human health are still not fully understood (Raghav et al. 2013).

The main sources of ECs are wastewater treatment plants (WWTPs) for domestic sewage, wastewater from hospital effluents, chemical manufacturing plants, livestock effluents and agriculture runoff (De la Cruz et al. 2012). These contaminants include, among others, products as human and veterinary pharmaceuticals, personal care products, steroid hormones and surfactant wastes (Petrović et al. 2003). Since these compounds are complex, biological treatment methods cannot guarantee their complete removal in sewage treatment, so they tend to be released in wastewater treatment facilities effluents, causing toxic effects in aquatic and terrestrial ecosystems (De la Cruz et al. 2012).

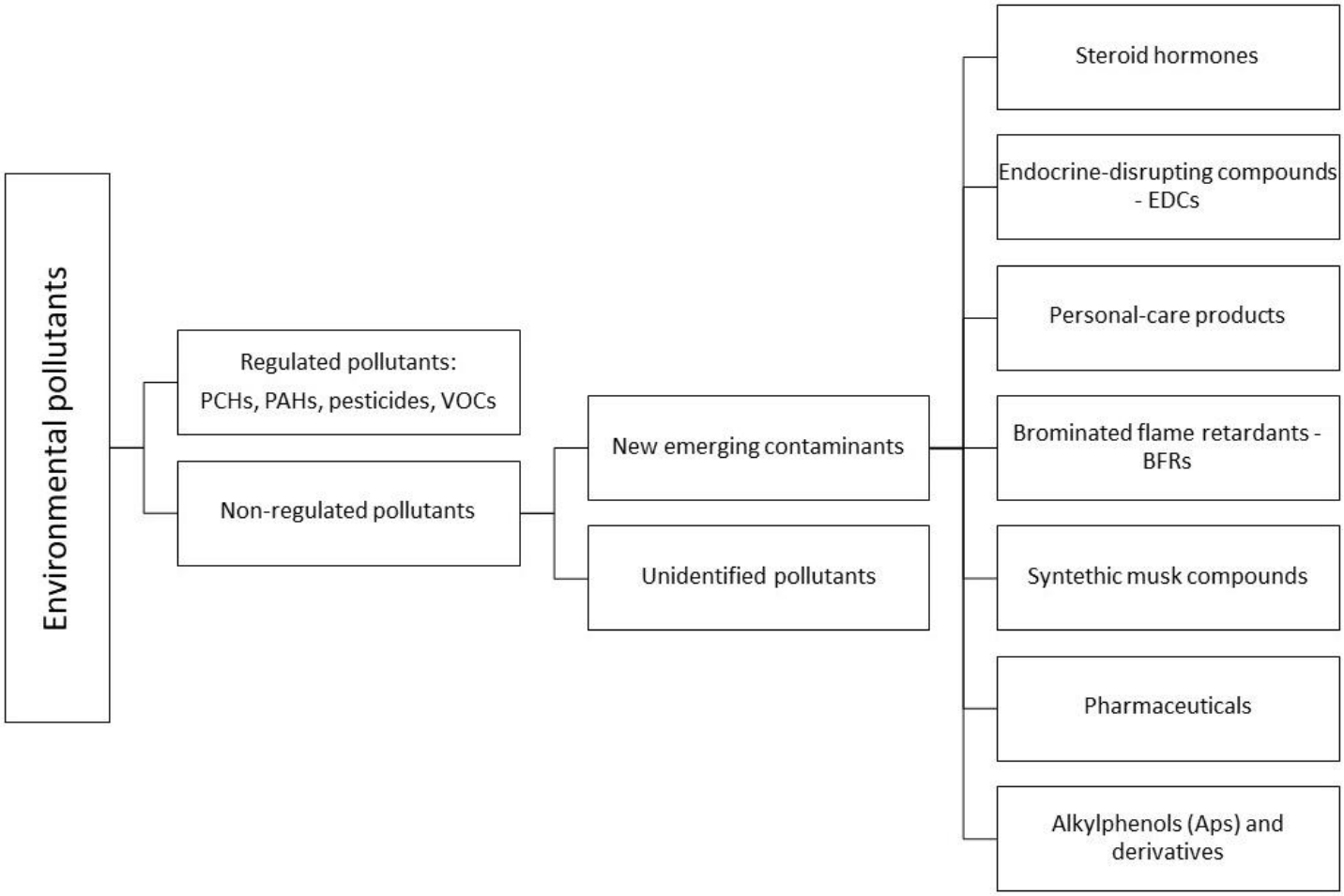

Figure 2.1 - Classification of contaminants. Adapted from Kot-Wasik et al. (2007).

According to Matamoros et al. (2012), ECs have been detected in many environmental compartments such as air, soil, surface water, and groundwater. Although the reported concentrations are generally low, these substances have been observed throughout the seasonal cycle in a variety of hydrological, climatic, and land-use settings (Kot-Wasik et al. 2007).

In this work, it will be given a special attention to pharmaceuticals. Pharmaceuticals are synthetic or natural chemicals used in medicines and are widely and gradually being utilized as a part of human and veterinary medication. They contain active components that have been designed to have pharmacological effects and confer significant benefits to society but also include compounds of environmental concern like antibiotics, legal and illicit drugs, analgesics, steroids, beta-blockers, etc (Gogoi et al. 2018). With the advance of analytical techniques for tracing pharmaceutical residues, many studies have demonstrated the widespread occurrence of pharmaceuticals in water environment, which lead to a higher concern on this matter to investigate the source, behavior, fate, risk, and control of such emerging pollutants (Li et al. 2014).

Moreover, the production and consumption of pharmaceuticals are increasing across both developed and developing countries, not only in terms of accessibility, but also the volume or

quantity of drugs consumed (Marsik et al. 2017). One of the factors contributing to this rise is a growing demand for drugs to treat aging-related diseases. However, the rise in pharmaceutical consumption is also observed in countries with younger populations, indicating that other factors, such as physicians' prescription habits, also play a role (OECD 2011). These aspects, together with the decreasing prices, is leading to the increase in consumption and availability of pharmaceuticals, making the ingesting of medicines a routine for most people.

Portugal follows this increase. According to Infarmed I.P. (2011), in the last years, an increase and a chronic consumption of several types of medicines have been observed in Portugal, the highest prescription and consumption regarding anxiolytics, hypnotics, antibiotics, lipid regulators, non-steroidal anti-inflammatories and analgesics.

Direcção Geral da Saúde (2017) concluded that, in general, Portuguese people are increasingly using antidepressants and antipsychotics and that the consumption of tranquillizers and medications to control hyperactivity in children and young people is also very high. In numbers, Portuguese people spent more than 30 million euros on medicines for depression, anxiety and other mental health problems (Shifter 2017). In 2016, almost 11,8 million packages of these types of drugs came out of pharmacies, more than double than in 2013 (about 5,6 million) (Shifter 2017). Another type of pharmaceuticals whose consumption has been increasing are the lipid-reducers. According to Infarmed I.P. 2014, in the last decade, the consumption of lipid-reducers more than doubled in Portugal.

This increase in the consumption can lead to higher levels of pharmaceuticals being released into the environment.

2.2 Pharmaceuticals analysed

In this work, two different commonly used pharmaceuticals, from two different types of medicines, were considered: bezafibrate and paroxetine (Table 2.1).

Table 2.1 – Chemical properties of bezafibrate and paroxetine (National Center for Biotechnology Information).

Medicine	CAS	Molecular formula	Molar weight (g/mol)	log k_{ow}
Bezafibrate	41859-67-0	$C_{19}H_{20}ClNO_4$	361,85	4.25
Paroxetine	61869-08-7	$C_{19}H_{20}FNO_3$	329,37	1.23 (pH 7)

Bezafibrate (Figure 2.2) is a substance that is part of the fibrate group of drugs, which are the type of pharmaceuticals used to treat hyperlipidemia. It is an antilipemic agent that lowers cholesterol and triglycerides, by decreasing low-density lipoproteins and increasing high-density lipoproteins (National Center for Biotechnology Information). Recently, bezafibrate was included in the list of the most used pharmaceuticals in the world (Dantas et al. 2007).

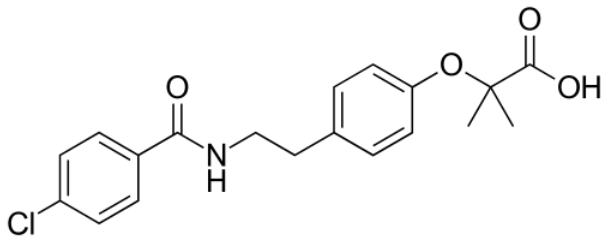

Figure 2.2 - Molecular structure of bezafibrate.

Paroxetine (Figure 2.3) is an antidepressant that works as a selective serotonin uptake inhibitor (SSRI). It has no active metabolites and has the highest specificity for serotonin receptors of all the SSRIs. This medicine is effective in the treatment of most depressive disorder, obsessive-compulsive disorder, social anxiety disorder, panic disorder, posttraumatic stress disorder, generalized anxiety disorder and premenstrual dysphoric disorder (National Center for Biotechnology Information).

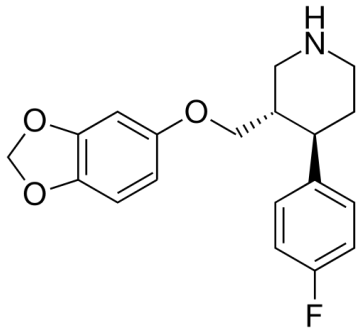

Figure 2.3 – Molecular structure of paroxetine.

2.3 Occurrence of pharmaceuticals in the environment

Since humans started to use chemicals to treat diseases, low levels of pharmaceuticals have been found in the aquatic environment. However, with the recent increase in the consumption and use of pharmaceuticals, a higher amount of pharmaceuticals residues and their metabolites are being released to the environment. There are various pathways for the contamination of water such as excretion of drugs by humans and animals, unused medicines disposed to the domestic sewage system, leaks from landfills, effluents from hospitals, runoff from animal husbandry and aquaculture sites, etc. (Pal et al. 2010).

However, the main source of this release into the environment is through the municipal wastewater treatment facilities (Lapworth et al. 2012). After administration, most pharmaceuticals are not completely metabolized when ingested by humans and animals, so these substances are excreted with urine and feces into the sewage system (Li et al. 2014). In Figure 2.4, the pathway for the release of contaminants through excretion to drinking water is represented.

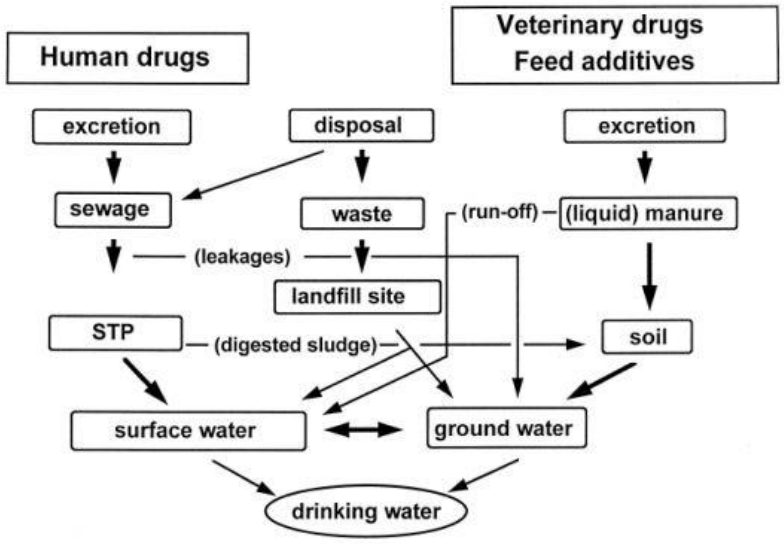

Figure 2.4 - Fate and transport of pharmaceuticals in the environment (World Health Organization 2011).

Another pathway for the release of pharmaceuticals is the unused and expired pharmaceuticals that people usually dispose in the normal household waste or discard into the sink or toilets, entering the sewage system (Zhang et al. 2008).

Also, to a minor extent but still relevant, the wastewater from hospitals or pharmaceutical manufacturers also increases the quantity of pharmaceuticals in municipal wastewater. The municipal wastewater then enters the WWTPs, but most of these facilities are not properly equipped to deal with these ECs and many compounds pass through conventional treatment systems without removal (Shaver 2011). Subsequently, the effluent from the WWTPs is discharged, along with the ECs, to surface waters (rivers, lakes and estuaries). Once in surface waters, pharmaceuticals have been shown to interrupt the natural biochemistry of many aquatic organisms including fish and algae (Shaver 2011).

Until now, there are no legal requirements for discharge of these ECs, but this scenario is expected to change in the next few years (Álvarez-Torrellas et al. 2018). The European Union (EU) has approved a watch list of 17 substances, including 7 pharmaceuticals, for their monitoring in the EU-water basins (Decision 2015/495/EC) (European Commission 2015). Due to this emerging concern, it is undoubtedly necessary to improve or/and find alternatives for the

treatment of contaminated effluents, preventing their discharge to the environment. It is also urgent to find methods to treat environments that are already contaminated.

2.3.1 Pharmaceuticals in Portuguese rivers and estuaries

The extensive urban activities along the Portuguese coast and main rivers lead to high aquatic contamination levels and consequent environmental and human exposure (Pereira et al. 2014). Due to these pressures, Portugal has been characterized as a moderate to high water stress area (Pereira et al. 2017).

According to Pereira et al. (2017), after evaluating the concentrations of 17 pharmaceuticals of different therapeutic groups in surface waters from 20 different sites across Portugal, it was confirmed the presence of 11 pharmaceuticals in Portuguese surface waters and each sample presented up to 8 pharmaceuticals. Concerning each therapeutic group, the mean concentrations from the different drugs of each type were, in decreasing order: SSRIs (37.9 ng/L), anti-inflammatories (35.9 ng/L), antibiotics (33.5 ng/L), antiepileptics (10.9 ng/L) and lipid regulators (9.4 ng/L).

López-Serna and Petrović (2012) for instance, reported values of pharmaceutical levels in one of most important rivers in Spain, Ebro River. The concentrations from the different groups of pharmaceuticals were: 26.71 ng/L of anti-inflammatories, 25.05 ng/L of lipid regulators, 12.86 ng/L of antihypertensives, 11.71 of antihistamines, 11.08 ng/L of antibiotics and 11.04 ng/L of psychiatric drugs, levels of the same order of magnitude as those in Portugal.

The occurrence of ECs in water is directly related to their removal in WWTPs. Regarding the effluents from WWTPs discharged in the rivers, and according to Pereira et al. (2014), after analyzing the influents and effluents of 15 different WWTPs (across Portugal), in two sampling campaigns (spring and summer), it was detected a mean concentration of 1369.4 ng/L of bezafibrate in the WWTP influents and 302.2 ng/L in the WWTP effluents, with a mean removal efficiency of 79,2%, which means most WWTPs were not so effective in the removal of this drug. The maximum concentration of bezafibrate detected in the WWTP effluents was 2400 ng/L.

On another study from Silva et al. (2014), it was evaluated the presence of four SSRIs (fluoxetine, paroxetine, sertraline and citalopram) in the influents and effluents of 15 different WWTPs across Portugal, in four sampling campaigns (spring, summer, autumn and winter). Fluoxetine and sertraline were only found in WWTPs influents, however paroxetine and citalopram were found in both WWTPs influents and effluents. According to the results, it was detected a mean concentration in WWTP influents of 170.0 ng/L of paroxetine and in WWTP effluents of 81.1 ng/L, corresponding to a mean removal of 80.37%.

Comparing the values from the Portuguese studies with studies from other countries (Tables

2.2 and 2.3), it is possible to verify that the Portuguese values are generally higher than the rest, however studies from Spain and Greece found similar values to the Portuguese ones. These differences may be explained by the different locations of the WWTPs, regarding not only the number of population and area that the treatment plant covers but also the industrial activities and hospitals nearby.

Table 2.2 - Concentration values of bezafibrate in wastewater treatment plants influents and effluents in different countries (ng/L).

Bezafibrate			
Country	**Influents**	**Effluents**	**Reference**
Portugal	1369.4	302.2	Pereira et al. 2014
Spain		40 - 110	Bueno et al. 2009
		50 - 130	
	206 - 424	5 - 26	Gros et al. 2012
Greece	245.2 - 755.9	27.4 - 233.8	Kosma et al. 2014
	n.d - 945.9	n.d - 344.2	
	n.d - 769.5	n.d - 278.2	
Italy		0.3 - 117	Castiglioni et al. 2005
	63 - 120	11 - 48	Verlicchi et al. 2012
Canada		11 - 260	Comeau et al. 2008

Table 2.3 - Concentration values of paroxetine in wastewater treatment plants influents and effluents in different countries (ng/L).

Paroxetine			
Country	**Influents**	**Effluents**	**Reference**
Portugal	170.0	81.1	Silva et al. 2014
Norway	0.6 - 12.3	0.5 - 1.6	Vasskog et al. 2006
	11.5	3.8	Vasskog et al. 2008
	2.9 - 12.9	1.0 - 11.7	
Spain	1649	89	Gros et al. 2012
Italy	20 - 80	10 - 18	Verlicchi et al. 2012
UK		6.6 - 9.8	David et al. 2018
Canada	1.8 - 16	1.3 - 12	Lajeunesse et al. 2012

These results highlight the importance of pharmaceuticals contamination in surface waters,

recognizing this issue as a priority for environmental strategies. These pollutants affect the rivers and subsequently, the estuaries, at the mouth of these rivers, that are rich ecosystems with an important biological and economical role (Aminot et al. 2016). There are various evidences of pharmaceuticals in estuaries (Thomas and Hilton 2004; Togola and Budzinski 2007; Madureira et al. 2010; Klosterhaus et al. 2013; Yan et al. 2015; Aminot et al. 2016; Cantwell et al. 2018). According to Thomas and Hilton (2004), of 14 pharmaceuticals monitored, 10 were found in water of 5 different estuaries in UK. Cantwell et al. (2018) found 8 pharmaceuticals of the 14 scanned in every sample of the Hudson river estuary in the USA. In a study done by Aminot et al. (2016), of the 53 monitored compounds, 36 were detected at least once and 18 were detected in more than 75% of the water samples from the estuary of Garonne river in France, one of the largest European estuaries. Among the pharmaceuticals detected in the referred estuaries are antihypertensives, antibiotics, anti-inflammatories and lipid regulators, including bezafibrate.

In the case of Portugal, and according to Madureira et al. (2010), all the 7 pharmaceuticals monitored were detected in water samples from the Douro river estuary. Although the highest concentration (178 ng/L for carbamazepine) detected in the Douro River estuary was lower than the lowest observed effect concentration (LOEC) (1 µg/L) described in a study by Triebskorn et al. (2007), the safety margin is not so big when considering a mixture of pharmaceuticals (Madureira et al. 2010). Madureira et al. (2010) also concluded that the results of the study indicate that other pharmaceuticals not considered in this study are present in the Douro river estuary and thus, there is a potential risk of mixtures of pharmaceuticals interfering with the aquatic species.This study also showed that pharmaceuticals in Douro River generally tend to have higher concentrations in the lower stretches, corresponding to the most urbanized areas, which means that the higher concentrations of pharmaceuticals are highly dependent on the location of WWTPs, on the influence of contaminated water from the tributaries and on the direct discharge of illegal untreated effluents (Madureira et al. 2010).

2.3.2 Techniques used in the analysis of the studied drugs in the environment

To find an analytical method to detect all pharmaceuticals is merely impossible. To detect low concentrations of the most pharmaceuticals, sophisticated analytical research methods with very low detection limits are necessary (Siddiqui et al. 2017).

When residue analysis of pharmaceuticals became an important issue in the 1990s, gas chromatography (GC) was the preferred chromatographic technique together with various derivatization procedures for the analytes (Buchberger 2007). Chromatography can be coupled to different types of detectors, such as a diode detector (DAD) or a mass spectrometer (MS) (Picó et al. 2017a). Undoubtedly, the application of advanced measurement technologies like gas chromatography with mass spectrometry (GC-MS) and GC with tandem MS (GC-MS^2) or liquid

chromatography with MS (LC-MS) and LC with tandem MS (LC-MS^2) for environmental analysis has allowed the determination of a broader range of compounds, including pharmaceuticals, and has therefore permitted more comprehensive assessment of environmental contaminants (Fatta et al. 2007). LC-MS^2 is becoming more commonly used in pharmaceuticals analysis because of its high sensitivity and its ability to confirm compounds identity, allowing separation and detection of compounds that have the same molecular mass but different product ions, even if they co-elute (Fatta et al. 2007).

These technological advances have improved the sensitivity and accuracy of detection equipment and analytical methods which can be noticed by the increase in studies showing very low concentrations of pharmaceuticals in various environmental matrices (Hubert et al. 2017), including surface water, groundwater, treated wastewater effluent and drinking water (World Health Organization 2011).

Chromatography allows an efficient separation of chemically similar compounds and consists on a separation based on the interactions of the solute with the mobile phase and the stationary phase (Figure 2.5).

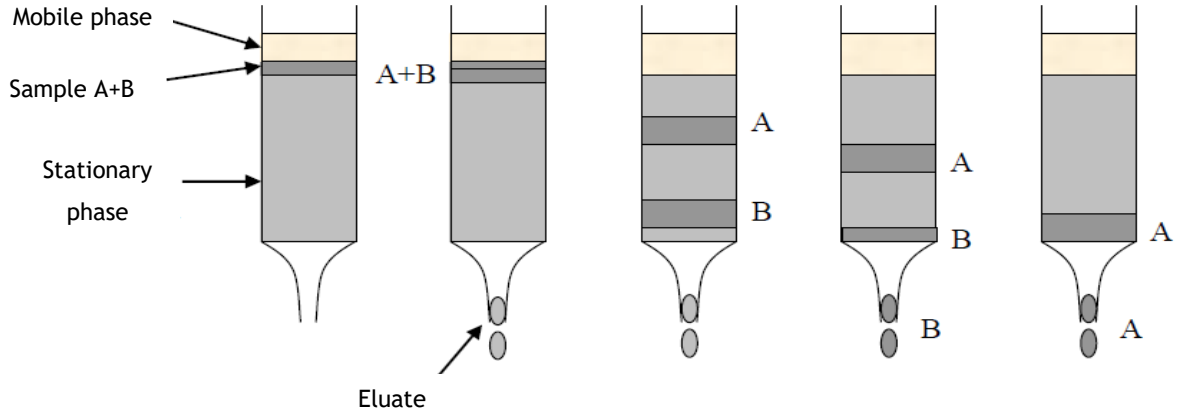

Figure 2.5 – Representation of the chromatographic separation of compounds.

However, in most cases, to detect low concentration values present in the environment, preconcentration and separation of the analytes from the matrix is a prerequisite for reaching a low detection limit. For this reason, sample-preparation procedure becomes one of the most important parts of the analysis of organic compounds in environmental matrices.

Solid-phase extraction (SPE) is, nowadays, considered the best technique for the fractionation/purification step, and it is one of the most used techniques to extract pharmaceuticals from the sample into a small volume of solvent (Fatta-Kassinos et al. 2010). Polymeric sorbents, mainly the hydrophilic-lipophilic balance (HLB) eluted with methanol, are the preferred system to clean-up pharmaceuticals and PCPs (Picó et al. 2017). SPE is simple, rapid and economic technique, based on a non-equilibrium, exhaustive removal of chemical

constituents from the flowing liquid sample (mobile phase) via retention on a contained solid sorbent and subsequent recovery of selected constituents by elution from the sorbent (stationary phase) (Matamoros et al. 2012). SPE is a well-established sample-preparation technique, with which a good sensitivity is obtained (Tong et al. 2009; Fatta-Kassinos et al. 2010).

2.4 Estuaries and salt-marshes

An estuary is a partially enclosed water body near the coast where freshwater from rivers and salt water from the ocean combine and mix (Potter et al. 2010); being a transition place between the coast and the ocean (Figure 2.6). Since estuaries have a mixing of waters with different types of salt concentrations, it allows them to create exceptional conditions, being able to support completely different organisms (Sun et al. 2012).

Estuaries are highly productive ecosystems that have an important place in biogeochemical cycles, however they are also very fragile ecosystems that suffer from high anthropogenic pressure receiving all type of contaminants (Fernandes et al. 2017a).

The sources for estuarine contaminations are storm drains, industrial discharges, runoff from lawns, streets and farmlands, discharges from sewage treatment plants, and atmospheric deposition (Sun et al. 2012). Most of these contaminants can be dissolved in water, accumulated in estuarine sediments and/or bioaccumulate in organisms (Sun et al. 2012), causing serious effects in several organisms, ecosystem degradation, habitats deterioration and possible human poisoning. For this reason, estuaries are considered sinks for contaminants, being imperative new remediation and recovery strategies (Fernandes et al. 2017a).

Figure 2.6 – Estuaries of Cávado River (A) and Douro River (B).

2.5 Phytoremediation

A possible method to recover and remediate contaminated environments is phytoremediation. Traditional physical and chemical methods used for the remediation of soil and water

contaminated with organic compounds are usually expensive and environmentally damaging (Afzal et al. 2014). Phytoremediation offers a good alternative to the traditional methods. This technology, based on the natural processes, uses plants and associated microorganisms to remove, accumulate, metabolize, absorb and/or degrade organic and inorganic pollutants from contaminated sites (soil, water and air) (Fernandes et al. 2017a). It is a cost-effective, promising and trustworthy technology and represents a sustainable solution to recover damaged ecosystems, such as estuarine areas (Feng et al. 2017; Fernandes et al. 2017b). The process to clean up contaminate sites occurs by the uptake or degradation of contaminants, that happens primarily through the plant root system which provides a vast surface area that absorbs and accumulates water and nutrients needed for its growth along with contaminants. Plant roots release organic and inorganic exudates in the rhizosphere causing changes at the soil-root interface which affects the number and activity of microorganisms, the availability of the contaminants and the aggregation and stability of the soil. Root exudates can increase or reduce (immobilize) the availability of pollutants in root zone of plants by changing soil characteristics, releasing organic substances, changing chemical composition and increasing the microbial activity. (Ahalya and Ramachandra 2004)

Phytoremediation can be used as a complementary technology, “working” along with others or as an alternative, for instance when conventional clean-up technologies require high capital inputs and are labor and energy intensive.

However, phytoremediation has some limitations, for example, it depends on the soil properties and on the level of contamination (Pilon-Smits 2005). It is confined to rooting depth and the remediation process could affect the food chain when chemicals are uptaken by the plant. Also, the treatment time, typically several years, is seen as the biggest disadvantage (Pilon-Smits 2005). Nevertheless, it is also an eco-friendly and natural solar-energy driven clean-up technology, based on the concept of using nature to clean nature.

Phytoremediation is a technology that is applicable to both organic and inorganic contaminants, present in solid substrates (sediments), liquid substrates (water), and air, and there are different types of phytoremediation techniques (Figure 2.7), according to the type and speciation of the pollutant, and to the characteristics of the plant species (Salt et al. 1998):

- Phytoextraction: extraction of pollutants and accumulation in plant tissues, followed by harvesting of the (above ground) plant material. The plant material can subsequently be used for nonfood purposes (e.g., wood, cardboard) or ashed, followed by disposal in a landfill or, in the case of valuable metals, recycling of the accumulated element (phytomining);
- Phytodegradation: the use of plants and their associated microorganisms for the degradation of organic pollutants in the root zone;

- Rhizofiltration: absorption and adsorption of pollutants by plants roots, from water and aqueous waste streams (e.g. plants can be used as filters in constructed wetlands (CWs));
- Phytostabilization: reduction of contaminants bioavailability by plants roots and subsequent retention in the root zone. By immobilizing the contaminants the process reduces leaching, controlls erosion and creates an aerobic environment in the root zone, adding organic matter to the substrate that can bind the contaminants. Microbial activity associated with the plant roots may accelerate the degradation of organic contaminants;
- Phytovolatilization: the use of plants to volatilize pollutants;
- Phytostimulation: plants facilitate biodegradation of organic pollutants by microbes in their rhizosphere;
- The use of plants to remove pollutants from air through filtration.

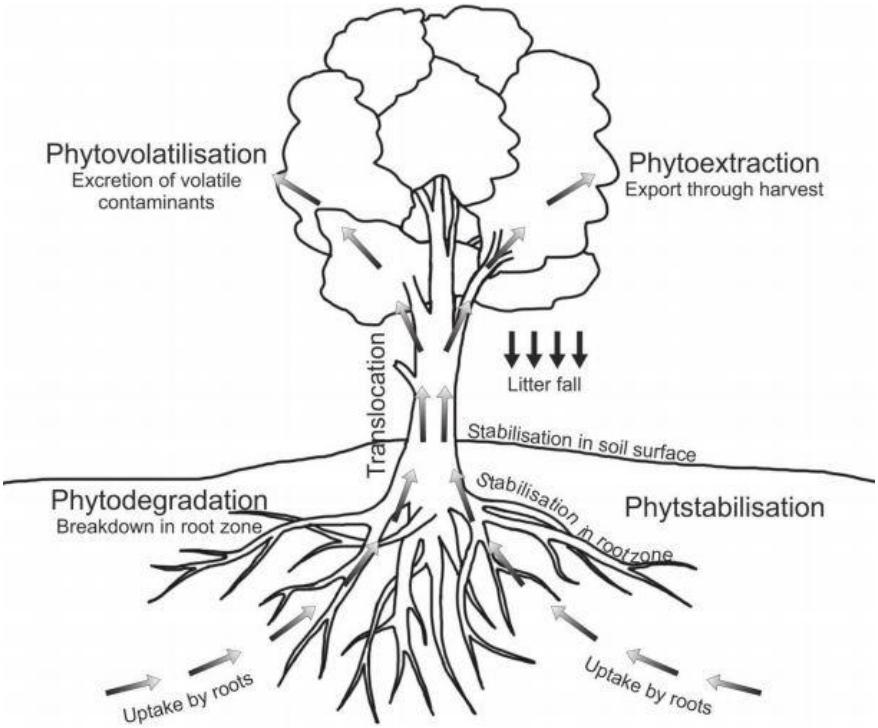

Figure 2.7 – Representation of the different types of phytoremediation. (Lenart-Boroń 2014)

These various phytoremediation technologies are not mutually exclusive. For instance, in a CW, accumulation, stabilization and volatilization can occur simultaneously (Pilon-Smits 2005).

As already mentioned, the different phytoremediation technologies described above are suitable for different classes of pollutants. For instance, one of the most common uses of phytoremediation is in the remediation of soils contaminated with metals. Phytoremediation of metals has been widely studied in the last years, including in estuarine environments

(Windham et al. 2003; Nunes da Silva et al. 2014; Teixeira et al. 2014; Oliveira et al. 2014a; Fernandes et al. 2017c). Plants have developed mechanisms to tolerate metal contamination, such as synthesis of metal binding peptides, vacuolar sequestration, immobilization of metals in cell walls, exclusion through the action of plasma membrane, phytovolatilization, among others (Teixeira et al. 2014). Phytoremediation of metals can occur by two main strategies: reduction of the metal mobility through absorption, adsorption, and/or precipitation by plant roots, hence decreasing their bioavailability (phytostabilization); and uptake of contaminants by plant roots and translocation to aboveground parts of the plant (phytoextraction) (Teixeira et al. 2014).

Phytoremediation has also been applied to organic pollutants. Feng et al. (2017) listed studies that used mechanisms of plant-endophyte bacteria phytoremediation of organic contaminants, for instance PAHs (Bisht et al. 2014), petroleum hydrocarbons (Oliveira et al. 2014b; Ribeiro et al. 2014; Kukla et al. 2014), pyrene (Sun et al. 2014), diesel (Yousaf et al. 2011), crude oil (Fatima et al. 2015), BTEX (Taghavi et al. 2005) and trichloroethylene (TCE) (Weyens et al. 2009, 2015).

The acceleration of pollutants removal by organisms, e.g. microbial communities, is commonly used as an "in situ" environmental friendly cleanup method for organic pollutants. There are two basic forms of microbial bioremediation: biostimulation (BS), i.e. the injection of nutrients to induce microbial propagation of the native microbial population; and bioaugmentation (BA), the addition of enriched microbial cultures, resistant to the pollutant, to enhance its degradation (Ribeiro et al. 2014).

More recently, studies reported the use of phytoremediation to treat contaminated environments by ECs, mostly pharmaceuticals and personal-care products (PPCPs). Dordio et al. (2009) evaluated the ability of Typha spp. to remove clofibric acid (lipid regulator pharmaceutical) from contaminated water. Typha spp. is an emergent macrophyte which has been frequently used to depurate water contaminated with organic compounds and has shown a good tolerance when exposed to some xenobiotic substances. Dordio et al. (2009) reported that Typha spp. was able to remove 80% of clofibric acid after 21 days of exposure to a solution spiked with 20 $\mu g\ L^{-1}$, with over 50% being removed just within the first 24–48 h. These results not only illustrate the potential of Typha to remove clofibric acid from contaminated water but also serve as a model for other pharmaceuticals or other organic xenobiotics with similar chemical properties, and thus suggest the potential use of constructed wetlands (CWs) planted with Typha for removing a wider range of related compounds from wastewaters.

In fact, another important application of phytoremediation is in constructed wetlands (Figure 2.8), constructed systems that mimic natural wetland processes. CWs have been studied to be very efficient for treatment of conventional pollutants in a variety of wastewaters such as domestic wastewater, agricultural wastewater, industrial effluent, mine drainage, leachate, contaminated groundwater and urban runoff. However, the treatment of waters contaminated with pharmaceutical contaminants using CWs, is still a recent application field (Li et al. 2014).

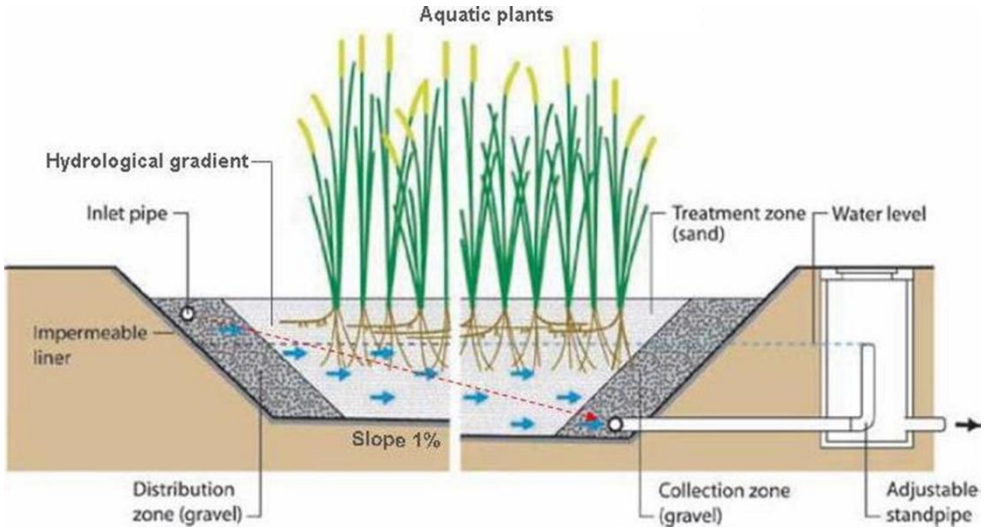

Figure 2.8 – Schematic representation of a constructed wetland. Adapted from Wang et al. (2017).

Zhang et al. (2014) and Chen et al. (2016) described the removal of PPCPs by means of aquatic plant-based systems such as CWs. According to Chen et al. (2016), after analyzing the occurrence and removal of 24 pharmaceuticals of different types in 3 CWs in Czech Republic, the author concluded that CWs with horizontal subsurface flow could be effective natural treatment systems for most of the pharmaceuticals, with at least one of the CWs showing removal efficiencies higher than 80% for all compound. Furthermore, this study indicated that the design and operation parameters of different CWs resulted in a large variability in the treatment efficiency of pharmaceuticals.

Zhang et al. (2014) gives an overview of the present state of researches on the removal of PPCPs in CWs and concluded that direct plant uptake of selected PPCPs has a major role in removal by hydroponic plant-based aquatic systems. These authors also concluded that it is worth noting that phytoremediation is considered particularly important for those pharmaceuticals which are relatively recalcitrant to biodegradation (e.g., clofibric acid), or are highly polar/soluble (e.g., caffeine).

2.5.1 Salt-marsh plants and potential of phytoremediation

Most estuaries present large salt marsh areas colonized by different plants, salt–tolerant plant

species - halophytes plants (native and invasive species) that are capable of completing their life cycle in salt concentration around 0.2 M NaCl or even higher and in an area subject to recurrent flooding (Oliveira et al., 2015). These salt marsh plants and their associated microbial communities are important in the process of removal organic and inorganic contaminants (Fernandes et al. 2017a), namely because they are able to oxidize the sediment through the movement of oxygen towards the roots or acidifying its rhizosphere through the release of root exudates (Almeida et al. 2011).

The possibility of using salt marsh plants to control pollution has been studied (Windham et al. 2003; Almeida et al. 2011; Nunes da Silva et al. 2014; Oliveira et al. 2014a; Fernandes et al. 2017b), these studies concluding that plants such as *Juncus maritimus, Phragmites australis, Spartina patens, Triglochin striata and Spartina alterniflora* have potential to treat estuaries contaminated with metals and hydrocarbons. Regarding metal contamination, Windham et al. (2003) refer that plant activities within salt marshes have shown to increase metal retention as plant uptake of dissolved metals can reduce the input of metals into estuarine waters. Also, metal sequestration in plant tissues may provide a long-term sink if contaminated tissues are afterward buried.

More recently, these salt-marsh plants have been studied to attenuate environments contaminated with ECs in a few studies (Carvalho et al. 2012, 2013; Sauvêtre and Schröder 2015; Fernandes et al. 2015). Fernandes et al. (2015) studied the response of a salt-marsh plant, Phragmites australis, to a contamination with a veterinary antibiotic, enrofloxacin (ENR), and evaluate its potential for the bioremediation of this emerging contaminant. The plant and the respective rhizosediment were exposed to ENR under different nutritional conditions in sediment elutriates, a simplified but realistic medium. Fernandes et al. (2015) verified that 95% of added ENR was still in the unplanted system whereas in the planted one only 5% of added ENR could be detected and concluded that salt marsh plant–microorganism association has a natural potential to attenuate antibiotic contamination and their effects in estuarine areas.

In the study from Sauvêtre and Schröder (2015) the objective of the work was to study the uptake and degradation mechanisms of carbamazepine, an antiepileptic pharmaceutical, by *Phragmites australis* and its endobacteria and to give recommendations for its enhanced removal by phytoremediation. The author isolated endophytic bacteria from plants exposed to medium contaminated with 5 mg/L carbamazepine (a concentration 20–80 times higher than those usually found in municipal sewage water) and at the end of the experiment concluded that plants were able to remove 90% of the initial concentration from nutrient media within 9 days.

Sauvêtre and Schröder (2015) also referred that these results could serve in the future to

improve knowledge regarding plant degradation of the antiepileptic drug in wetland-based WWTPs.

Overall, salt-marsh plants have demonstrated potential to be used in phytoremediation techniques to treat different kinds of pollutants, but studies concerning the ones of emerging concern are still scarce. It is, therefore, of high-importance to evaluate the potential of these salt marsh plants to remove different pharmaceuticals, namely from estuarine areas to recover areas impacted by ECs.

2.5.2 Phytoremediation in co-contaminated environments

Sites polluted with pharmaceuticals are also frequently polluted with other chemicals of different nature, like, for instance, petroleum hydrocarbons, pesticides and surfactants and also inorganic pollutants such as metals (Almeida et al. 2008). The simultaneous presence of such wide variety of pollutants may constrain phytoremediation processes. The response of plants to the surrounding chemical environment and contaminants bioavailability may be influenced by the presence of different chemicals. Moreover, information about the possible occurrence of antagonisms and synergisms effects among different pollutants simultaneously present in the sediment, and whether and how such phenomena influence plant-microorganisms interactions are still not fully understood (Mucha et al. 2011).

Several studies showed that the presence of organic and inorganic contaminants may influence the response of the salt marsh communities and, consequently, the phytoremediation process (Almeida et al. 2008, 2009; Mucha et al. 2011; Moreira et al. 2013).Therefore, to evaluate the phytoremediation potential it is important to take into account the different contaminants present in the aquatic environments.

2.6 Objectives of the study

This study aims to explore the application of phytoremediation for the removal of pharmaceuticals from estuarine areas, using autochthonous a salt marsh plant, being divided in two different objectives.

Firstly, a survey of the presence of two pharmaceutical compounds, a psychiatric drug (paroxetine) and an anti-lipid drug (bezafibrate) in different estuarine sediments, non-vegetated or vegetated with different salt marsh plants, such as *Phragmites australis*, *Juncus maritimus* or *Halimione portulacoides*, were initially carried out.

In second place, controlled laboratory experiments were carried out to evaluate the potentialities of a salt marsh plant, *Phragmites australis*, and the microorganisms associated to its roots (rhizospheric microorganisms) to remove the selected pharmaceutical compounds, including in the presence of other estuarine contaminants such as metals (namely copper),

simulating the different types of contaminants that can be found in the estuaries. Degradation rates were evaluated by measuring the pharmaceutical compounds in both solutions and sediments through chromatographic techniques.

This salt-marsh plant was chosen due to its potential of phytoremediation of different types of pharmaceuticals demonstrated in previous studies, such as Sauvêtre and Schröder (2015) and Fernandes et al. (2015).

3 Materials and Methods

3.1 Sample collection and preparation for determination of pharmaceuticals in estuarine sediments

In the first field collection, on the 21st of February 2018, sediments in contact with roots of salt marsh plants (rhizosediment) and sediments from non-vegetated areas (distance from plants were about 5 m) were collected at two estuaries in Portugal.

The first point (Point 1) was in River Cávado Estuary (41.523247, -8.785919) in northern Portugal (Figure 3.1). Two samples of sediments were collected, rhizosediment from the salt marsh plant *Haliminone Portulacoides* and non-vegetated sediment.

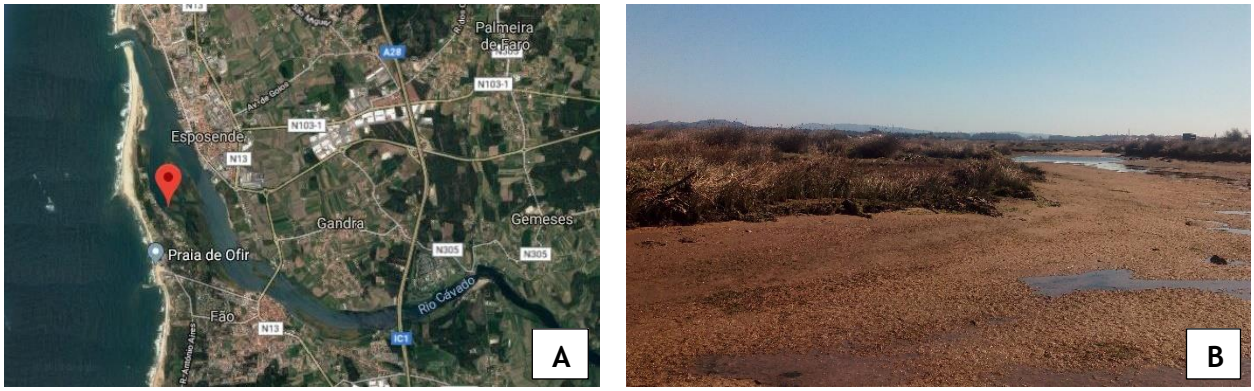

Figure 3.1 - A: Location of Point 1. B: Picture of the River Cávado Estuary.

The second point (Point 2) was in River Lima Estuary (41.689822, -8.816289) in north of Portugal (Figure 3.2). Two samples were also collected, rhizosediment from *Phragmites australis* and non-vegetated sediment.

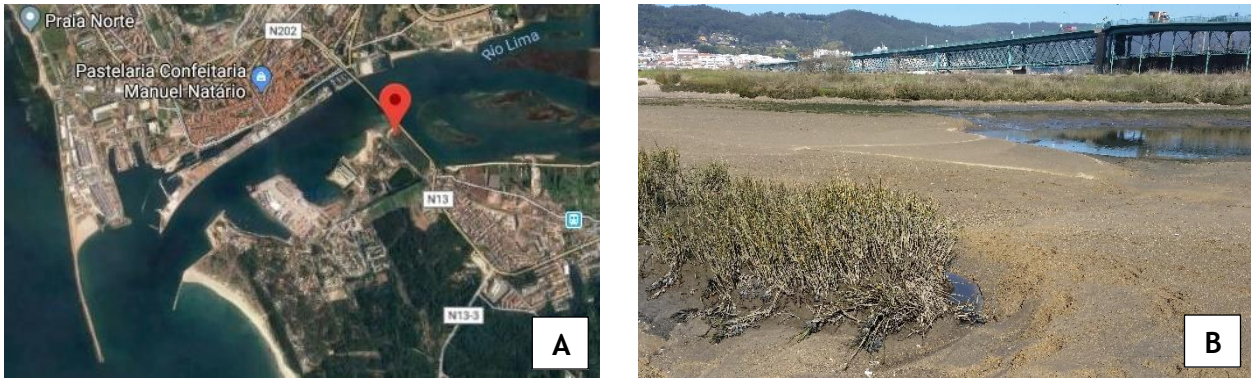

Figure 3.2 – A: Location of Point 2. B: Picture of the River Lima Estuary.

The third point (Point 3) was in another part of the River Lima Estuary (41.688905, -8.793773), near Darque (Figure 3.3). Two samples were also collected, rhizosediment from *Juncus maritimus* and non-vegetated sediment.

Figure 3.3 – A: Location of Point 3. B: Picture of the River Lima Estuary, near Darque.

In the laboratory, the samples were homogenized and stored at -20°C during 24 hours for posterior lyophilization. After three days in the freeze dryer, the sediments were grinded using a mortar and pestle and then passed through a sieve of 2 mm. The sediments under 2 mm were stored in aluminum foil until extraction and analysis by chromatography.

3.2 Evaluation of *Phragmites australis* phytoremediation potential

Plants (*P. australis*) and the respective rhizosediment (sediment around plant roots) were collected in River Lima Estuary (41.689822, -8.816289) on the 22nd of March 2018 in the north of Portugal. Estuarine water was also collected at this point.

In the laboratory, rhizosediment was homogenized, and large stones and remains of plant tissues were removed. Then elutriates were prepared accordingly to the protocol of EPA (US EPA 1991).Elutriate were prepared by mixing in each flask 50 g of sediment with 200 mL of estuarine water. The flasks were manually shaken to remove soil clods and placed on a shaker for 30 minutes. After that, flasks were left to settle during 24h. In total, 50 flasks were prepared and divided in two groups: one left with the sediment for experiments with sediment plus elutriate and another for experiments only with elutriate. For the latter, after 24h, solutions were filtrated through 0.45 µm pore size filter (cellulose nitrate membrane, Millipore). The plants *(P. australis)* were washed with deionized water and inserted in 15 glass flasks with sediment and 15 glass flasks without sediment, 3 plants per flask.

The system was assembled like in Figure 3.4. The system was divided in 5 different treatments: copper, bezafibrate, paroxetine, copper+bezafibrate and copper+paroxetine

Afterwards the flasks were doped with the respective contaminants, 200 µL of a 10 g/L solution of copper chloride (attaining a 100 mg/L copper concentration in solution), 20 µL of a 1 g/L methanolic solution of bezafibrate (attaining a 100 µg/L bezafibrate concentration in solution) and 20 µL of a 1 g/L methanolic solution of paroxetine (attaining a 100 µg/L paroxetine

concentration in solution).

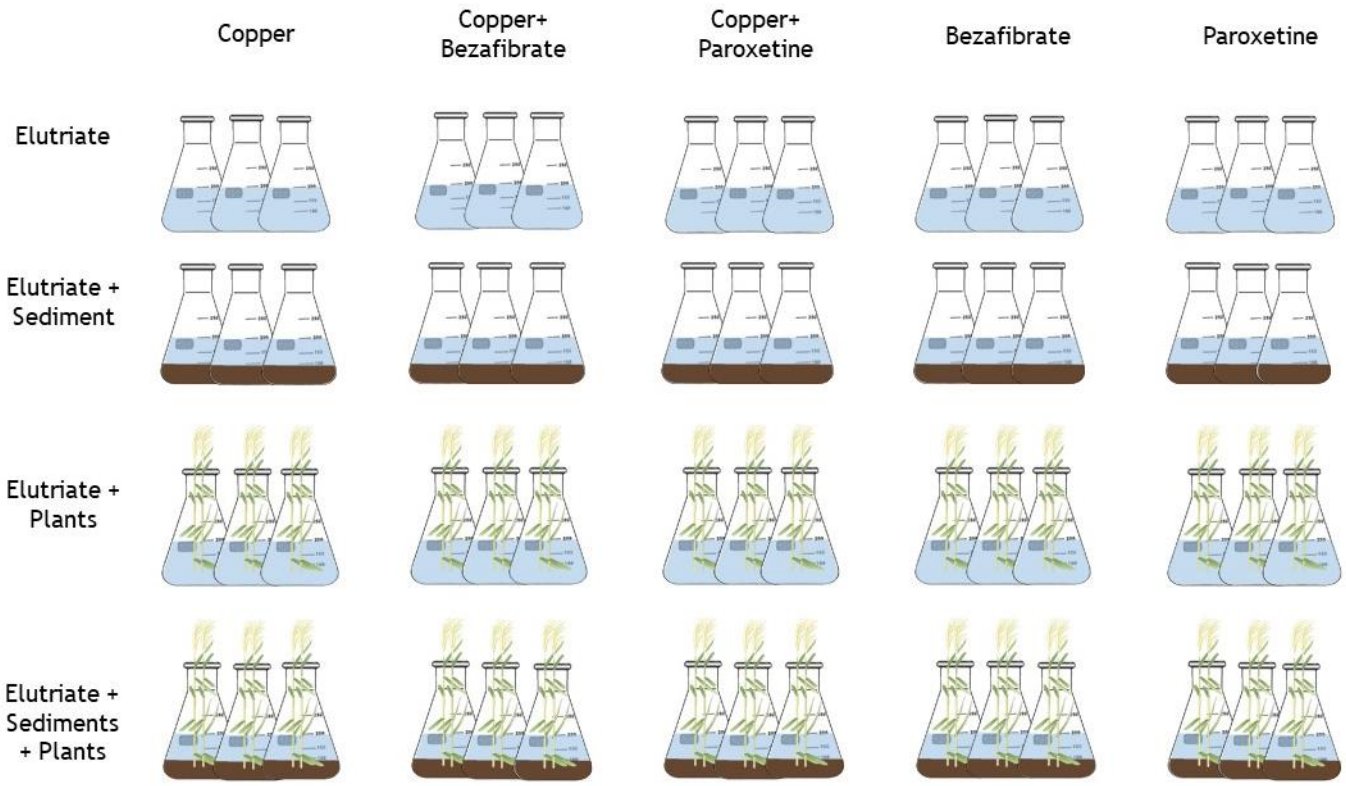

Figure 3.4 - Representation of the experimental assembly

Each flask was wrapped in aluminum foil to avoid light degradation (Figure 3.5). The flasks were exposed to natural day: night regime with natural sunlight for one week (7 days). During the week, a second (third day) and a third (fifth day) doping of 100 µg/L of bezafibrate and paroxetine was performed.

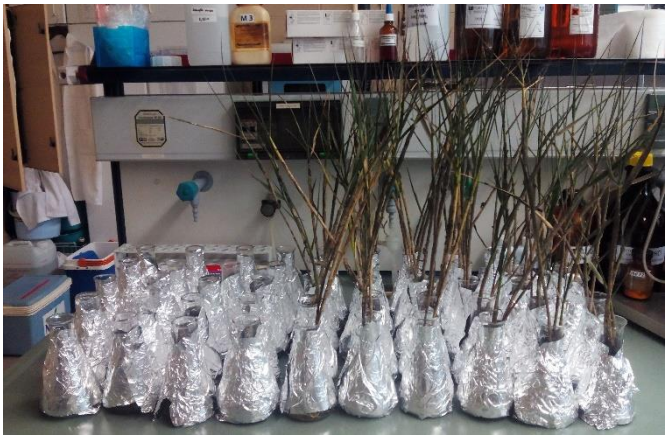

Figure 3.5 – Flasks during the week of experiment.

The flasks were doped three times, to simulate a continuous discharge of pollutants instead of one single discharge, and to evaluate the behavior of the plant during the whole week of experiment exposed to the pharmaceuticals. The total doping was of 0.3 mg/L of bezafibrate

and paroxetine, which is a much higher concentration than what is found in rivers and WWTP effluents. This concentration was chosen to simulate a worst-case scenario, big discharge of contaminants, exposing the plant to an extreme situation.

The flasks with copper were doped only at the beginning of the experiment, since it is an inorganic compound which is not removed from the system, contrary to organic contaminats that can be degraded.

At the end of the experiment, 15 mL of elutriate samples were collected from each flask and stored at - 20 ºC until analysis. Sediment samples were collected from each flask and also stored at -20ºC for lyophilization.

3.3 Analysis of pharmaceuticals

3.3.1 Materials and reagents

To prevent contamination, all sampling and labware materials were soaked in 20 % (v/v) HNO_3 solution for at least 24 h, rinsed several times with bi-deionised water (conductivity<0.1 mS cm -1) and dried in a Class 100 laminar flow hood. The sample manipulation was carried out in a clean room with Class 100 filtered air. All reagents used were pro analysis grade or equivalent.

3.3.2 Standard "stock" solutions

Initially 10 mg of bezafibrate or paroxetine were weighed, then dissolved in 10 mL of methanol, giving a concentration of 1 g/L for each pharmaceutical. All these stock solutions were placed in amber vials and stored at -20 °C.

3.3.3 Standard working and daily solutions

The standard working solutions were prepared from the individual standard stock solutions attaining concentrations of 5 mg/L and 50 mg/L of bezafibrate and of paroxetine in methanol. All these solutions were also placed in amber vials and kept at -20 °C.

Standard daily solutions were prepared every day with concentrations between 0.1 and 5 mg/L (Table 3.1). These daily standard solutions were prepared immediately before analysis in methanol and the mobile phase (H_2O/formic acid 99:1 v/v) used in the analytical equipment (25:75; v/v).

Table 3.1 - Daily standard solutions of bezafibrate and paroxetine; (a) – solution of 50 mg/L of bezafibrate and paroxetine was used instead of 5 mg/L.

Concentration (mg/L)	$V_{Solution\ 5\ mg/L}$ (µL)	V_{MeOH} (µL)	$V_{Mobile\ phase}$ (µL)
0.1	20	180	800
0.2	40	160	800
0.4	80	120	800
0.6	120	80	800
0.8	160	40	800
1.0	200	0	800
1.5	300	0	700
2.0	400	0	600
5.0	100 [(a)]	100	800

3.3.4 Ultrasonic bath extraction

For sediments extraction an ultrasonic bath extraction was carried out, based on a previously optimized methodology (Talaya 2015) and summarized in Appendix 1. For that, 1 g of sediment was accurately weight in amber vials and 5 mL of a solution of methanol and ammonia (95:5; v/v) was added. The extraction was done in triplicate for each type of sediment. The vials were then placed in an ultrasonic bath (Transsonic 460/H) for 15 minutes, with a foam support. The solutions were afterwards centrifugate (Selecta Mixtasel) for 5 minutes at 2500 rpm (Figure 3.6).

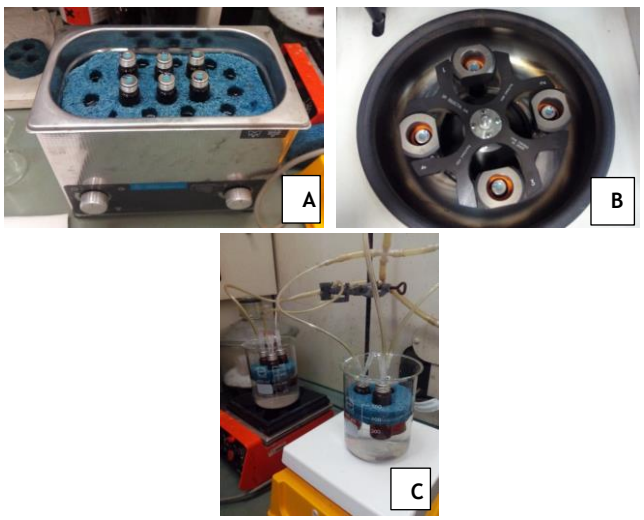

Figure 3.6 - Several steps of sediment extraction. A: Ultrasonic bath. B: Centrifugate. C: Evaporation by N2 flux.

After this process, all the supernatant was collected to another vial and 5 mL of

methanol/ammonia solution (95:5; v/v) were added again to the remaining sediment. The same procedure was applied, so each sediment was subjected to two sequential extractions. The two supernatants were combined and evaporated until dryness by a N_2 flux: 2 holes were made in the septum of each vial, in which pipette tips were inserted, then the vials were emerged in a water bath at approximately 30ºC (Figure 3.6). After the solvents evaporation, the residue was dissolved with 200 µL of methanol and 800 µL of mobile phase (water / formic acid, 99:1, v/v). The samples were stored in the freezer at -20ºC until analysis. Before analysis, one of the triplicates was divided and one part was doped with a solution of 5 mg/L of bezafibrate and paroxetine to check the analysis accuracy, attaining a concentration of 0,5 mg/L of bezafibrate and paroxetine.

3.3.5 SPE extraction

SPE was performed to concentrate the bezafibrate and paroxetine present in solutions collected from the experiment and to clean the matrix. For the SPE extraction cartridges Oasis MCX (3mL, 3cc) from Waters Corporation (Millford, MA, USA) were used in the ManiFold vacuum system (Supelco, Spain) (Figure 3.7).

Figure 3.7 – SPE procedure.

The SPE procedure carried out was based on a previously optimized methodology (Sousa 2014) and summarized in Appendix 1. Firstly, the cartridges were conditioned with 5 mL of methanol, followed by 5 mL of deionized water. Then the samples were loaded. Afterwards, the cartridges were cleaned with 5 mL of methanol/water (5:95 v/v) and left to dry for 30 minutes. For the elution, for bezafibrate 5 mL of methanol/formic acid (96:4 v/v) solution were used. For paroxetine, elution was carried out with 5 mL methanol/ammonia (95:5 v/v).

To evaluate the best eluent for each pharmaceutical with the SPE procedure, three aqueous standard solutions with a concentration of 0.5 mg/L of bezafibrate and paroxetine were prepared. Subsquently, the SPE procedure was carried out for each standard solution, using a

three different eluents: methanol/formic acid (96:4 v/v), methanol and methanol/ammonia (95:5 v/v).

After this initial test, to evaluate if the recoveries of each pharmaceutical were constant along the study, aqueous standard solutions with a concentration of 0.5 mg/L of bezafibrate and paroxetine were analyzed together with the elutriated samples. Then the SPE procedure was carried out, following the previously optimized methodology. Afterwards, SPE extracts were evaporated by a N_2 flux in a bath at approximately 30 ºC. Once the solvents have been evaporated, the residue was dissolved with 200 µL of methanol and 800 µL of mobile phase (water/formic acid 99:1, v/v). The samples were stored in the freezer at -20ºC until analysis.

Additionally, to evaluate if the recoveries of the SPE procedure were the same for the elutriates, 4 mL from every sample of elutriate were doped with 100 µL of the solution of 5 mg/L of bezafibrate and paroxetine, attaining a concentration of 0.125 mg/L, being afterwards subjected to the SPE methodology.

3.3.6 High-performance Liquid Chromatography

Bezafibrate and paroxetine were analyzed in a Beckman Coulter equipment (HPLC-system gold) using a previously optimized methodology (Sousa 2014) and summarized in Appendix 1. The equipment was provided with a diode array detector (DAD) (module 128) and an automatic sampler (module 508). The column was a 150 mm × 4.6 mm C18 Kinetec column (Phenomenex, UK).

Two mobile phases (water/formic acid, 99:1, v/v) and acetonitrile (both always degassed for 15 minutes in the ultrasound) were used. The gradient used was 100% of eluent A (water-formic acid, 99:1, v/v) for 1 min, followed by a 13 min gradient to 100% of eluent B (acetonitrile). Then, 100% of eluent A was reached again in 1 min to restore the column. Flow rate gradient started with 1 mL min^{-1}, which was maintained for 1 min, then it decreased to 0.8 mL min^{-1} for 13 min and then increased to 1 mL min^{-1}.

The sample injection volume was set at 50 µL and the detector signal was monitored at λ = 298 nm for paroxetine and λ = 252 nm for bezafibrate.

A calibration was performed using a mixture solution of paroxetine and bezafibrate of 5 mg/L, methanol and a mobile phase (water/formic acid 99:1) with the daily stock standard solutions mention in section 3.3.3. This calibration was carried out every day.

After the calibration, 1 mL of each sample was transferred to the HPLC vials and the analysis was performed.

3.4 Statistical analysis

Each estuarine sediment, rhizosediment and non-vegetated sediment were analyzed in triplicate, being the mean and standard deviation calculated afterwards.

In the case of the experiment fo the evaluation of *Phragmites australis* phytoremediation potential, elutriate and sediment samples of the different treatments were analyzed for the respective compound. Each sample was treated independently, being the mean and standard deviation calculated.

For pharmaceutical concentrations significant ($p < 0.05$) differences among samples were evaluated through a parametric one-way analysis of variance (ANOVA).

4 Results and discussion

4.1 Optimization and validation of the analytical methodology

4.1.1 Calibration curve

A calibration was carried out every day with the daily stock standard solutions presented at Table 3.1. An example of a calibration curve is represented in Figure 4.1.

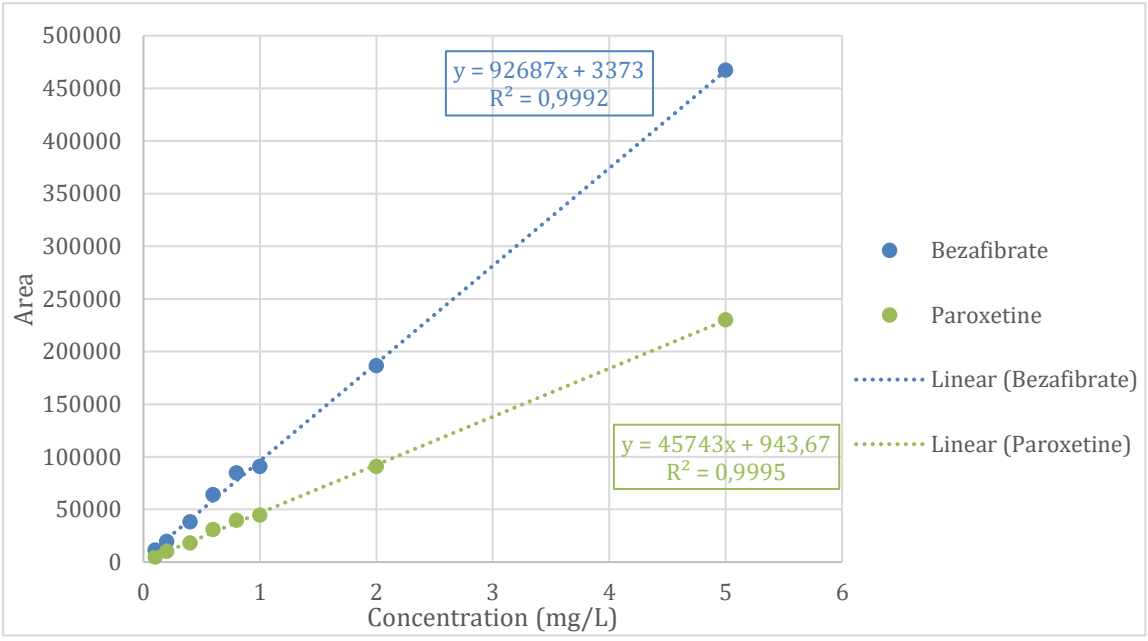

Figure 4.1 - Example of calibration curve for bezafibrate and paroxetine.

Analyzes of standard solutions over the time period of these studies resulted in calibration lines similar to those given as example.

4.1.2 Recovery of each pharmaceutical in SPE procedure

Three aqueous standard solution with a concentration of 0.5 mg/L of each pharmaceutical were tested using the SPE procedure with the different eluents, to evaluate the recovery for each pharmaceutical. After the chromatographic analysis and comparing the concentration values with the initial concentration (0.5 mg/L of bezafibrate and paroxetine), it was possible to evaluate the recovery of the SPE procedure for each eluent (Figure 4.2).

Analyzing the results, it is possible to conclude that the best eluent for bezafibrate is a solution of methanol/formic acid (96:4 v/v) with a recovery of 83%, and the best eluent for paroxetine is a methanol/NH_3 (95:5 v/v) solution with a recovery of 50%.

The results of the elution with methanol are not present because the concentrations were below detection limit ($LOD_{Bezafibrate}$ = 0.054 mg/L; $LOD_{Paroxetine}$ = 0.051 mg/L), therefore are not

significative.

Subsequently, all the elutriate extractions were performed taking into account these recoveries and following the same SPE procedure.

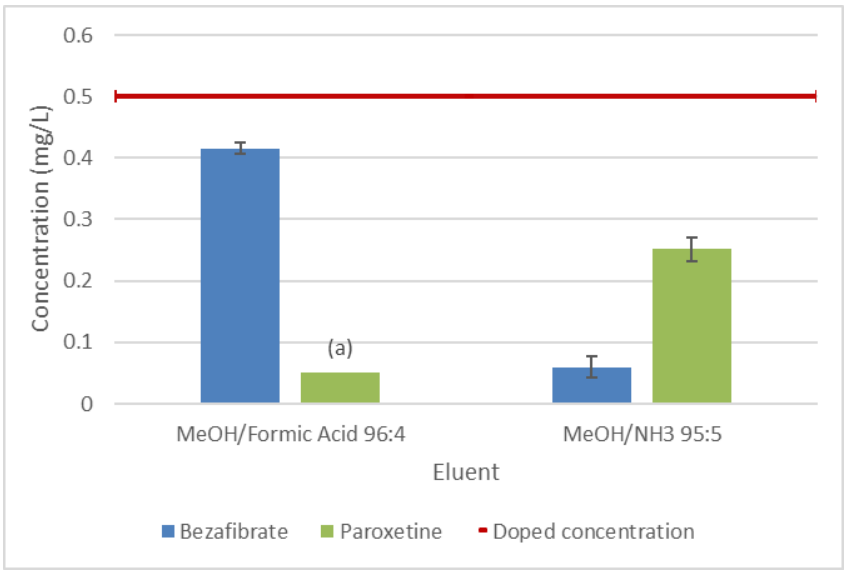

Figure 4.3 - Concentrations of bezafibrate and paroxetine in the aqueous standard solutions after the SPE procedure with different eluents. (a) - Value below detection limit ($LOD_{Bezafibrate}$ = 0.054 mg/L; $LOD_{Paroxetine}$ = 0.051 mg/L).

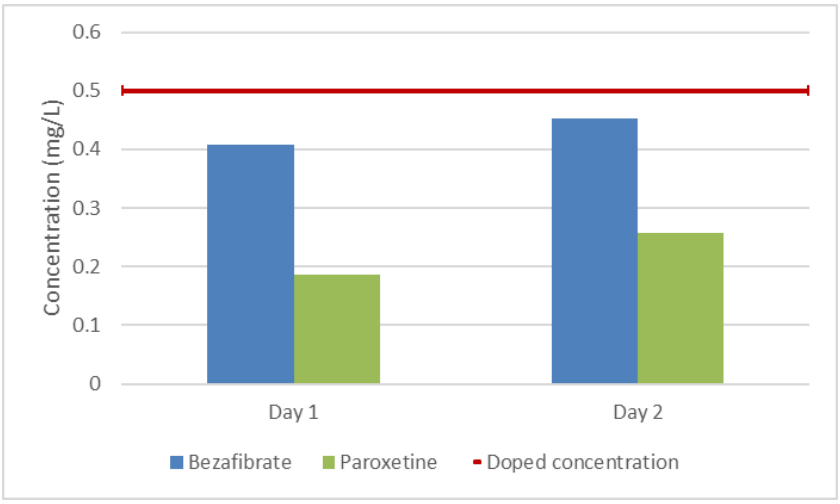

Figure 4.2 - Concentrations of the aqueous standard solutions of 0.5 mg/L of bezafibrate and paroxetine, along the study time.

To control this SPE recovery, along with the analyzed elutriate samples, an aqueous standard solution with 0.5 mg/L concentration of each pharmaceutical was also analyzed (Figure 4.3).

The results show a recovery percentage of 82% and 91% for bezafibrate, similar to the one previously obtained. In the case of paroxetine, recoveries of 37% and 52% were obtained, also similar to the values previously obtained.

4.1.2.1 Analytical accuracy of elutriate solutions extraction in SPE

As mentioned before, to evaluate the SPE procedure for the elutriates, 4 mL from every sample of elutriate were doped with 100 µL of the solution of 5 mg/L of bezafibrate and paroxetine, attaining a concentration of 0.125 mg/L, and subjected to SPE. The recoveries of the doped elutriates are presented in Figures 4.4 and 4.5.

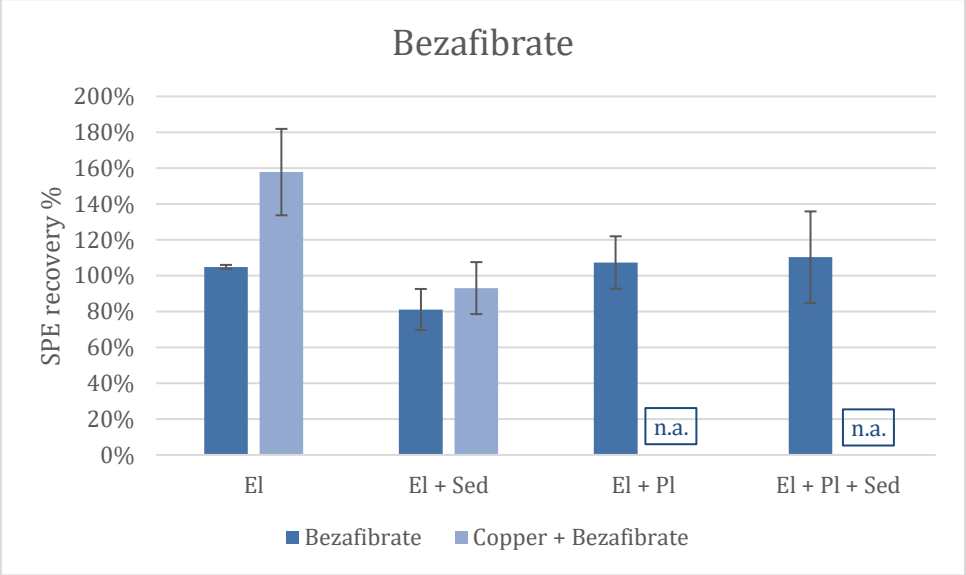

Figure 4.4 - Percentage of recovery of bezafibrate in the doped elutriate samples after SPE procedure. n.a. – not analyzed.

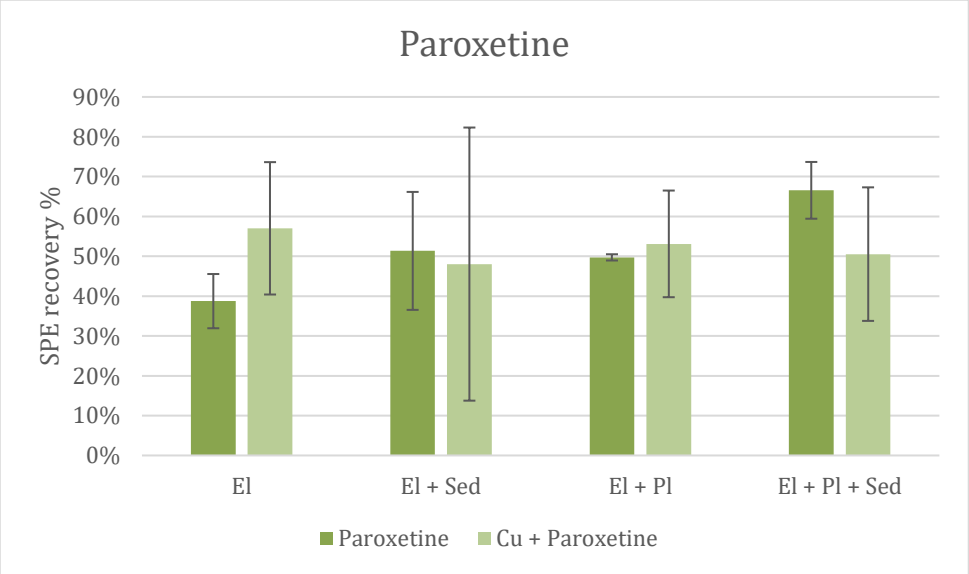

Figure 4.5 - Percentage of recovery of paroxetine in the doped elutriate samples after SPE procedure.

Considering the results presented in Figure 4.4, the recoveries of bezafibrate ranged between 81% and 110% similar to the percentage of recovery for the aqueous standard solutions (82%, 83% and 91%). The only exception was the sample of elutriate in the treatment with copper +

bezafibrate that presents a recovery of 158%, probably due to an analytical error.

Regarding the recoveries of paroxetine (Figure 4.5), the values ranged between 39% and 67%, being in accordance with the recoveries of the aqueous standard solutions (37%, 50% and 52%).

4.1.3 Analytical accuracy of sediment extracts analysis

For each sediment, after the extraction procedure, one of the triplicates was divided and one part was doped with the standard working solution of 5 mg/L, attaining a concentration of 0.5 mg/L of each pharmaceutical. The results regarding the concentrations of these doped samples extracts are presented in Figures 4.6 and 4.7. The recovery percentages were calculated attending to the amount of compound already present in the sediment.

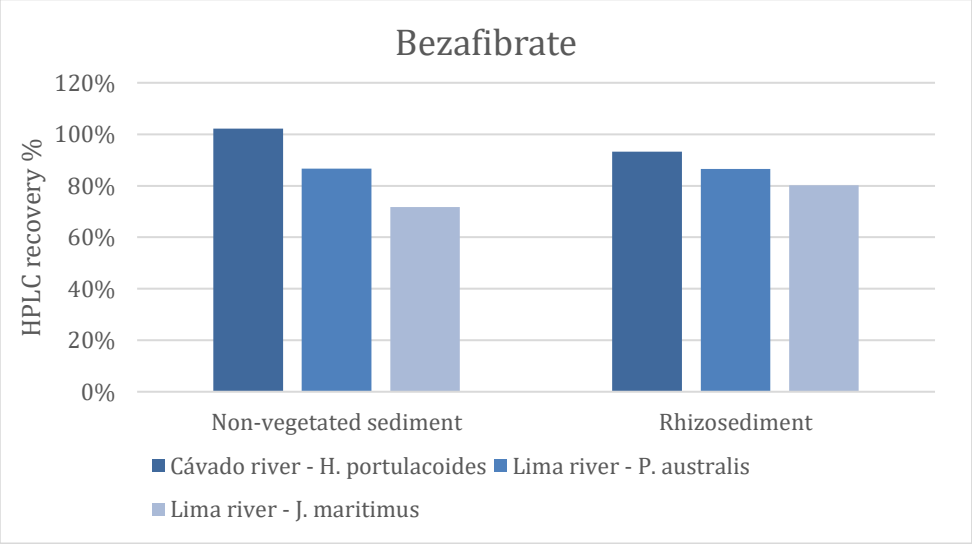

Figure 4.6 - Recovery of bezafibrate in doped sediment extracts after HPLC analysis.

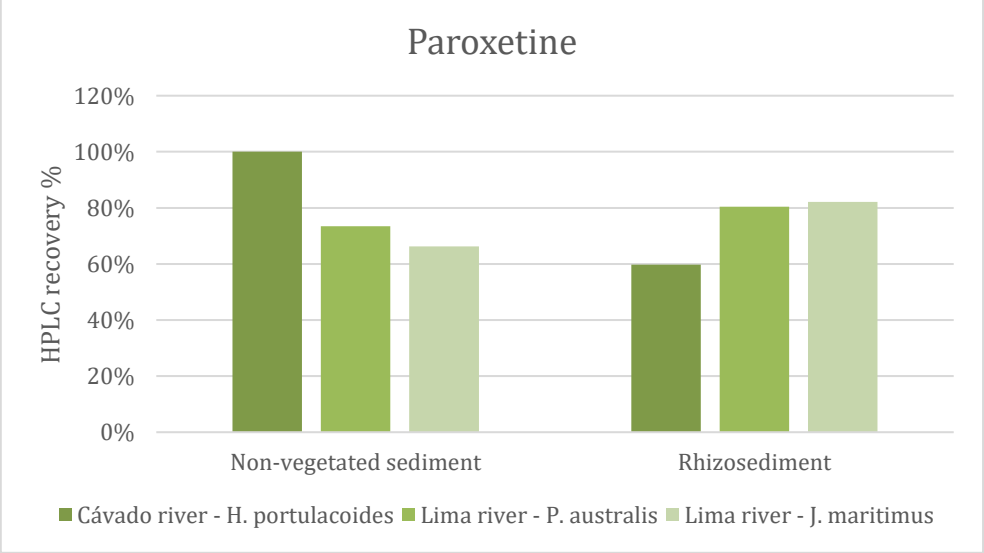

Figure 4.7 - Recovery of paroxetine in doped sediment extracts after HPLC analysis.

Recovery, for both non-vegetated and vegetated sediments, ranged between 72% and 102% for bezafibrate and between 60% and 100% for paroxetine. In general, the mean recovery of all extract samples was within the range 80-120% indicating that the HPLC calibration with aqueous standards solutions was suitable for quantifying the pharmaceuticals in sediments.

4.2 Determination of pharmaceuticals in estuarine sediments

Sediments from 3 points in estuaries in the North of Portugal were analyzed, including rhizosediment from three different salt-marsh plants and non-vegetated sediment close by each rhizosediment. In Figures 4.8 and 4.9 the results obtained for the three points are presented.

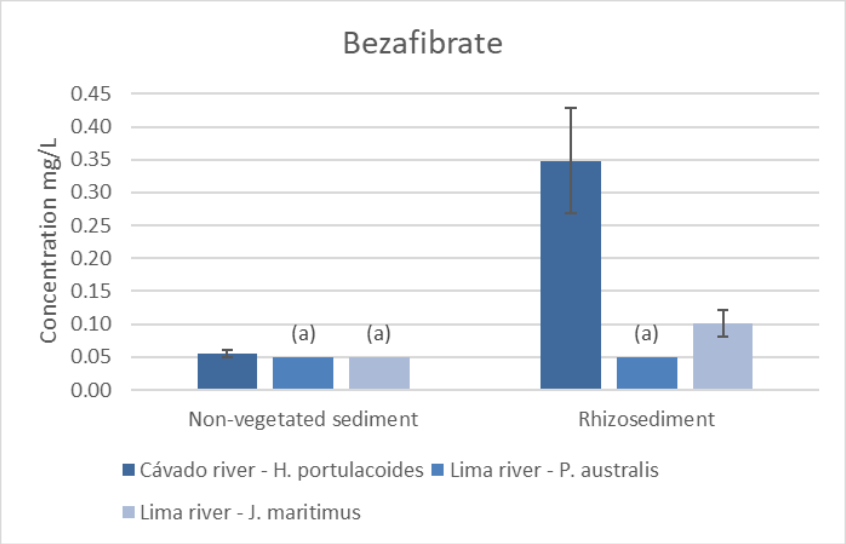

Figure 4.8 - Concentrations of bezafibrate in the different estuarine sediments. (a) - Value below detection limit (LOD = 0.054 µg/g).

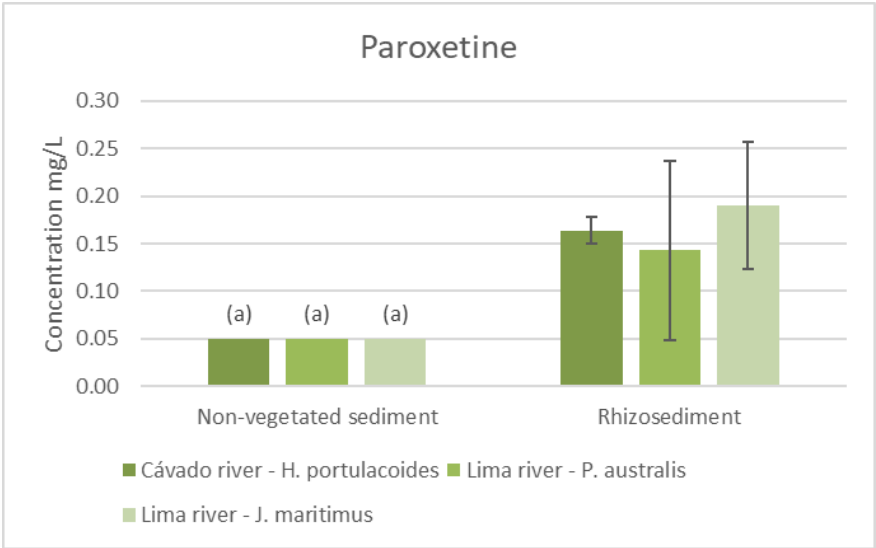

Figure 4.9 - Concentrations of paroxetine in the different estuarine sediments. (a) - Value below detection limit (LOD = 0.051 µg/g).

The results show that in non-vegetated sediments from Cávado River estuary no bezafibrate or paroxetine were detected. The concentrations levels in these sediments were below the detection limit. However, in the rhizosediment from the plant *Halimione portulacoides* from this estuary, both pharmaceuticals were detected, showing a concentration of 0.35 μg/g of bezafibrate and 0.16 μg/g of paroxetine.

Regarding sediments from Lima river estuary, the concentrations were similar in the two locations: in non-vegetated sediments none of the pharmaceuticals were detected and in rhizosediments from the plants *Phragmites australis* and *Juncus maritimus* none or low concentration of bezafibrate and concentration of 0.14 and 0.19 μg/g of paroxetine were found.

From the estuarine sediments and rhizosediments of these two estuaries it is possible to observe that rhizosediments had, in general, higher pharmaceuticals concentrations. On the other hand, in sediments non-vegetated neither of the studied drugs were detected. Therefore, it is possible to conclude the plants – *Halimione portulacoides*, *Phragmites australis* and *Juncus maritimus* – contributed, in general, to retain the contaminants in the sediment around roots. This has been also observed for other contaminants, such as metals and hydrocarbons (Almeida et al. 2011; Ribeiro et al. 2014; Oliveira et al. 2014a; Fernandes et al. 2017a, b).

4.3 Evaluation of *Phragmites australis* phytoremediation potential

After one week of experiment, all systems were disassembled. In the flasks with elutriate, plant and sediment, the roots of some plants were darker (almost black), which may indicate the beginning of the systems decomposition (Figure 4.10). The plants appeared to be on stress probably due to the experimental conditions, which was also pointed out by Carvalho et al. (2012) for this type of experiment.

Figure 4.10 - Different flasks after one week of experiment.

As mentioned before, the experiment had different treatments: elutriate (El), elutriate + sediment (El+Sed), elutriate + plant (El+Pl) and elutriate + plant + sediment (El+Pl+Sed). The plant tested was *Phragmites australis*, collected in Lima river estuary.

4.3.1 Removal of bezafibrate

Removal efficiency of the pharmaceuticals after one week of experiment was evaluated by measuring bezafibrate in elutriate solution and sediment. The respective results are present below.

4.3.1.1 Elutriate solution

To evaluate the removal efficiency of the system, each elutriate solution was collected and analyzed after the week of experiment, the results of the concentrations of bezafibrate being presented in Figure 4.11.

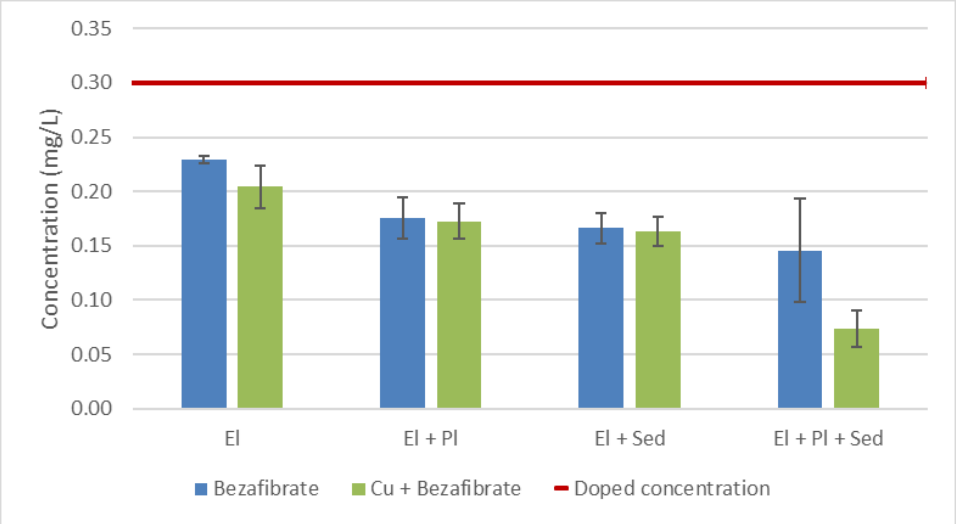

Figure 4.11 - Concentration of bezafibrate in elutriate of the different treatment.

Considering bezafibrate, it is possible to verify that this compound was partially degraded or retained. The flasks with only elutriate (El) had the higher concentration of bezafibrate in solution (ca. 0.23 mg/L), which corresponds to a removal of 23% of the bezafibrate added (0.3 mg/L). Since the elutriate was filtered, and therefore there were not a significant number of microorganisms, this percentage of removal is probably related with abiotic factors. Yang et al. (2011) reported that the most used methods of water sample preparation involves separating solid phases from water using membrane filters (e.g. pore size of 0.45 μm). However, the "dissolved" phase obtained includes complex fractions such as colloids of different sizes that present a large surface site density and large surface area. Thus colloids may present an enhanced sorption affinity for organic compounds such as pharmaceuticals (Yang et al. 2011). In the case of this study, a membrane filter of 0.45 μm was used to filter the elutriate, so

bezafibrate could have been aggregated to colloidal matter. Another abiotic process is photodegradation, Trovó et al. (2008) reported that solar radiation may favor the degradation of bezafibrate, however the flasks were wrapped in aluminum foil therefore significant photodegradation of the compound was not expected.

In the case of the treatment with elutriate + plant (El+Pl), the concentration of bezafibrate was ca. 0.18 mg/L, with a removal efficiency of 42%, indicating that the plant contributed for the removal of the compound.

When sediments were present, the concentrations after the week of experiment were lower than that in the flasks with only elutriate. A reduction of about 45% of bezafibrate was observed, with a concentration of ca. 0.17 mg/L, indicating that the compounds were probably retained or degrade in the sediment where the native estuarine microbial community was present.

The values in the two previous cases may be explained by the value of log K_{OW} of bezafibrate (log K_{OW} = 4.25). The diffusion process of organic compounds into the plant depends on their concentration, water solubility and hydrophobicity (expressed by log K_{OW}) (Dordio and Carvalho 2013). Organic compounds with moderate hydrophobicity (0.5 < log K_{OW} < 3) are considered easily taken up by the plants, while extremely hydrophobic compounds (log K_{OW} > 3) are tightly bound to soil organic matter, such as plant and animal residues (Carvalho et al. 2012). Since bezafibrate has a value of log K_{OW} of 4.25, it has a tendency to adsorb to the sediments and to the plant residues present in the sediments. So, removal from elutriate could be due to adsorption to sediment or, in the case of no sediment, adsorption to plant tissues (namely plant roots). However, in this last case a plant uptake (when no sediment was present) cannot be excluded.

Considering the treatment combining plant and sediments (El+Pl+Sed), the concentration of bezafibrate halved ca. 0.15 mg/L comparing to the initial one, however the difference between this concentration and the concentration in the treatment with only sediments was not significant, so it is not possible to understand the role of the plant in the presence of sediments.

Regarding the flasks doped with copper and bezafibrate, the results showed, in general, no significant differences among these and the treatment with only bezafibrate, except for the the system with elutriate + plant + sediments. In this case, the concentration showed a significantly higher reduction (ca. 0.07 mg/L), showing a removal of about 75% of the pharmaceutical, which means that the combination of the plant with the rhizosediment improved the degradation of bezafibrate. This indicates that the presence of copper (representing the different contaminants present in estuaries) might influence the retention or degradation of the compounds when all the components of the salt marsh estuarine system (water, plants and sediment) are present. Almeida et al. (2009) reported that some organic

pollutants may influence the phytoremediation of copper by *Halimione portulacoides*, so the reverse situation must be taken into account.

Overall, the presence of the salt-marsh plant and its rhizosediment showed a significant importance in the removal of bezafibrate.

4.3.1.2 Sediments

Bezafibrate concentration was also measured in sediment samples to evaluate the quantity associated with this matrix. As already mentioned, bezafibrate can present strong sorption to soils and sediments, due to its log K_{OW}. However, regarding the concentration of bezafibrate in sediments, values below detection limit ($LOD_{Bezafibrate}$ = 0.054 μg/g) were obtained in all treatments, therefore it was not possible to identify significant differences between treatments, nor confirm adsorption to sediments. Nevertheless, one cannot exclude the possible degradation of the drug in the sediment by the rhizospheric microorganisms that were present, as if all bezafibrate concentration removed from the elutriate solution would be adsorbed in sediment, the analytical methodology would allow its detection in sediments.

4.3.2 Removal of paroxetine

Removal efficiency of paroxetine was evaluated following the same procedure of bezafibrate, by measuring the compound in elutriate solution and sediment. The respective results are present below.

4.3.2.1 Elutriate solutions

After the week of experiment, elutriate solutions of the treatment with paroxetine were analyzed (Figure 4.12).

Since the mean recovery of paroxetine in the SPE procedure was of ca. 50%, the concentrations obtained were corrected in order to have more realistic values.

Regarding the flasks with paroxetine, it is possible to observe that the different treatments had removals of paroxetine of ca. 50% or more. In the case of the flasks with elutriate (El), after the week of experiment, paroxetine had a concentration of ca. 0.16 mg/L, which represents a removal of 47% of the compound. As already mentioned, some authors reported that the elutriate, despite being filtrated, still presents colloids forms, which exhibit a sorption affinity for pharmaceuticals (Yang et al. 2011). So, this percentage of removal may due to adsorption to colloids, although other abiotic factors might not be excluded.

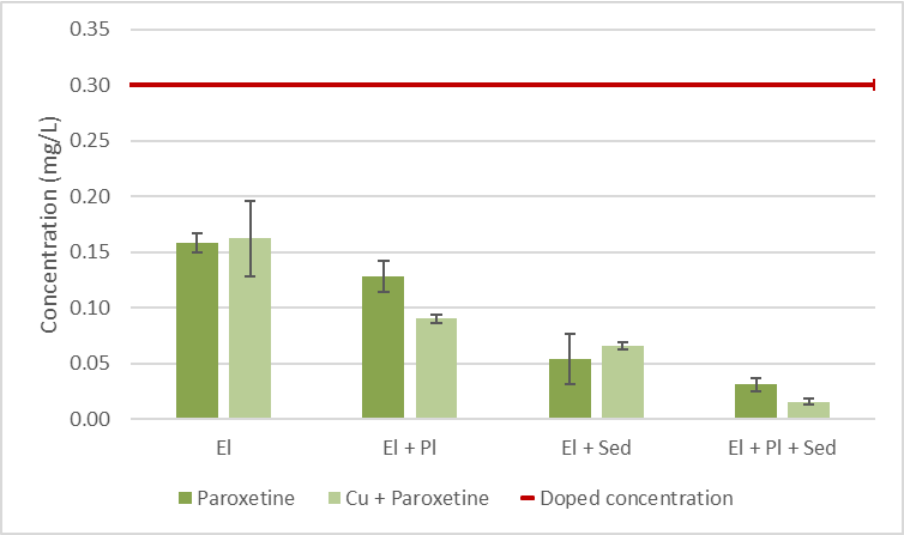

Figure 4.12 - Concentration of paroxetine in elutriate of the different treatment.

When sediments are considered (El+Sed), the removal efficiency increased significantly. The flasks containing elutriate + sediment present a concentration of 0.05 mg/L of paroxetine, when compared to the added concentration of 0.3 mg/L, represents a removal of 82%. In this case, both adsorption to colloids of the elutriate and sorption to the sediments and plant residues present in the sediments have to be taken into account. Moreover, microbial degradation by native microorganisms present in the rhizosediment should be considered as this compound can be biologically degraded (unpublished work).

In the case of the flasks with elutriate + plant (El+Pl), comparing with the treatment with only elutriate, the concentration of paroxetine decreases to ca. 0.12 mg/L which denotes a higher removal of the compound, with a percentage of 57%. Paroxetine could both adsorb to plant roots and be taken up by the plant, since *Phragmites australis* has been reported to uptake pharmaceuticals (Sauvêtre and Schröder 2015). Studies showed that the uptake of organic compounds by *P. australis* is related to the log K_{OW} and pK_a of the compound, being higher with compounds where log K_{OW} is between 1 and 3 (Schröder et al. 2008). Since paroxetine has a value of K_{OW} of 1.23, it can be easily taken up by the plants.

Considering the treatment with both sediment and plant (El+Pl+Sed), a significantly removal of paroxetine is observed. After the week of experiment, the concentration of paroxetine was ca. 0.03 mg/L, comparing with the added concentration (0.3 mg/L) represents a 90% of removal of the pharmaceutical. However, when comparing this value with the treatment with elutriate + sediment, similar concentrations were presented which indicates that once again it is not possible to understand the role of the plant in the presence of sediments.

Regarding the treatment with paroxetine and copper, in general, no significant differences relatively to the absence of copper were observed. The only exception was again for the

complete system, water+plant+sediment, and also for the treatment with elutriate+plant, both showing slightly higher removals.

4.3.2.2 Sediments

After the week of experiment, paroxetine concentration was measured in sediment samples of each flask to evaluate the quantity associated with this matrix (Figure 4.13).

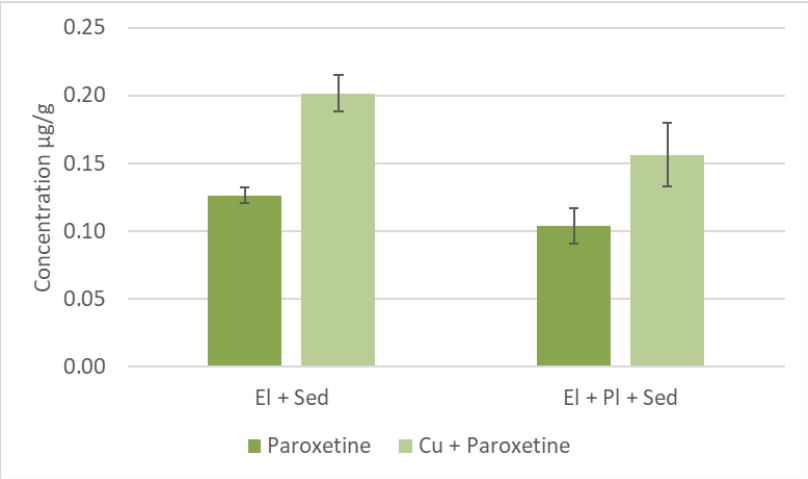

Figure 4.13 - Concentration of paroxetine in sediments samples of the different treatments.

The sediments doped with paroxetine and without plant (El+Sed), present a concentration of paroxetine of ca. 0.13 µg/g. In the case of the sediments in the treatment with plant (El+Pl+Sed), paroxetine concentration was similar, ca. 0.10 µg/g. So, in this case, contrary to bezafibrate, sorption to sediments had a significant effect on the removal of the compound from elutriate solution.

Regarding the sediments of the flasks with copper and paroxetine, the concentrations of paroxetine were slightly higher, with values of ca. 0.20 µg/g for El+Sed and 0.16 µg/g for El+Pl+Sed. These concentrations indicate that the presence of copper promoted a higher retention of the compound in the sediment, probably justifying the slightly higher removal from the elutriate solution as discussed above.

Considering that in total 300 µg/L of paroxetine were added to each flask (with 200 mL of elutriate). This means that each flask contained initially 60 µg of paroxetine, that would be distributed between the elutriate and the sediment. Regarding the paroxetine present in the elutriate solution, for instance, in the treatment El+Sed of ca. 0.05 mg/L, represents a quantity of 10.8 µg of paroxetine in the 200 mL of elutriate, which means that the remaining paroxetine should be distributed in the 50 g of sediment (each flask had 50 g of sediment and 200 mL of elutriate). Assuming a 50% of water in sediments, the theoretical value of paroxetine present in 1 g of sediment would be ca. 2 µg/g. Considering the value obtained in the analysis (ca. 0.13

μg/g), a substantial difference can be observed. This may indicate paroxetine degradation by the microorganisms present in the rhizosediments. There are several studies reporting the potential of microorganisms to degrade or remove different types of contaminants, including pharmaceuticals (Yu et al. 2006) and since rhizosediments present a large variety of microorganisms, the degradation of the compound by the microorganisms must be taken into account.

5 Conclusion

The presence of bezafibrate and paroxetine in different estuarine sediments, non-vegetated or vegetated with different salt marsh plants, *Phragmites australis*, *Juncus maritimus* and *Halimione portulacoides*, was evaluated. Both pharmaceuticals were detected in the rhizosediments analyzed, however, none of the compounds was detected in non-vegetated sediments. This indicates that the plant had a role in the distribution of these compounds in estuarine areas.

Afterwards, a controlled laboratory experiment was carried out to evaluate the potential of *Phragmites australis* (a salt-marsh plant) and the microorganisms associated to their roots (rhizospheric microorganisms) to degrade the selected pharmaceutical compounds, including in the presence of copper to simulate the different types of contaminants that can be found in the estuaries. Regarding the results obtained, in the systems without copper, the plant and its rhizosediment, when separated, showed a removal of 42% and 45% for bezafibrate and of 57% and 82% for paroxetine. When combined, a removal efficiency of 51% for bezafibrate and 90% for paroxetine was observed. This indicates that rhizosediment and the associated microorganisms have potential to degrade the pharmaceuticals. When combined with the salt-marsh plant, the percentages of removal do not show big differences, however the plants could have promote the degradation by microorganisms.

In the systems with copper, the plant and its rhizosediment, when separated, showed a removal of 43% and 46% for bezafibrate and of 70% and 89% for paroxetine and when combined, presented a removal efficiency of 75% for bezafibrate and 95% for paroxetine, which demonstrates that in some cases the presence of copper may influence positively the removal of the compound.

Overall, this study shows that the salt-marsh plant and specially it rhizosediments and the microorganisms associated have potential to remove the selected pharmaceutical compounds from estuarine environment and eventually degrade these contaminants, a feature that requires more research. The results also show the potential of *P. australis* to be used in CWs for the reduction/removal of the pharmaceuticals analyzed.

This study also shows that the estuarie environment (plant, sediments and water) have a natural potential to remove, retain and degrade emerging contaminants, so it is important to enhance this remediation.

6 Assessment of the work done

6.1 Objectives Achieved

The objectives of this study were:

- To carry out a survey of the presence of two pharmaceutical compounds, a psychiatric drug (paroxetine) and an anti-lipid drug (bezafibrate) in different estuarine sediments, non-vegetated or vegetated with different salt marsh plants, such as *Phragmites australis*, *Juncus maritimus* or *Halimione portulacoides*. This objective was fully accomplished.
- Evaluate the potentialities of *Phragmites australis* and the microorganisms associated to their roots (rhizospheric microorganisms) to degrade the selected pharmaceutical compounds, including in the presence of other estuarine contaminant such as metals, simulating the different types of contaminants that can be found in the estuaries, in controlled laboratory experiments. This objective was also fully achieved.

6.2 Other Work Carried Out

In parallel to this study, the potential of *Phragmites australis* to retain and uptake copper was also evaluated.

6.3 Limitations and Future Work

One limitation of this study was that the laboratory experience is a simplistic model with respect to what is happening in the estuary, additionally the input of pharmaceuticals in the environment is continuous and the response of the salt marsh community can be different. Another limitation, had to do with the technical part, the HPLC did not always work as expected, which delayed the analyzes.

In future work several changes can be applied to evaluate the performance of the system, such as, for instace, adding nutrients to the treatments which can improve the degradation by microorganisms. This study could also be done with different plants, since there are other salt-marsh plants that may have the potential to degrade contaminants, as described in the results. Other pharmaceuticals and contaminants can be tested to evaluate the behavior and efficiency of the plant.

It would be also interesting to evaluate the concentration of fluoride and chloride ions in solution since these compounds are released when paroxetine and bezafibrate are degraded, respectively, so they can be good indicators of the degradation of these pharmaceuticals.

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Appendix 1 Methodology

1.1 Optimization of analytical methods for determination of emerging contaminants (pharmaceuticals) in water

In the work of Ana Filipa Sousa de Prata (2014), a method for the determination of 3 pharmaceuticals in water was optimized. The pharmaceuticals were two lipid regulators (bezafibrate and simvastatin) and an antidepressant (paroxetine), compounds that have already been detected in wastewater, rivers and groundwater.

The analytical methodology included the pre-concentration of the samples through solid-phase extraction (SPE), followed by a high-performance liquid chromatography (HPLC), with diode array detector, analysis.

1.1.1 SPE extraction

First, two types of cartridges were tested, Oasis HLB (60mg, 3cc) and Oasis MCX (60mg, 3cc), using the ManiFold vacuum system (Supelco, Spain). The characteristic procedure for this type of cartridges was used, however, to verify the occurrence of losses or whether the compounds were effectively retained in the cartridges, sample/standard solutions were also collected after passage through the cartridge and analyzed.

In the experiments two pH values were tested: 2 and 7, for HLB cartridges.

Throughout the procedure, glass material was used to prevent loss of compound by adsorption to the material. Both pharmaceutical showed adsorption to plastic material (between 4 and 42%).

The procedure used for both HLB and MCX cartridges was, first, conditioning of the cartridges with 5 mL of methanol followed by 5 mL of deionized water and then the samples were passed (acidified to pH 2 or 7) through the cartridges. Thereafter, the cleaning step was performed by passing 5 mL of a methanol / water mixture (5:95 v/v) and letting cartridges to dry in vacuo for 30 minutes. Subsequently, one (HLB cartridges) or three sequential (MCX cartridges) elutions were performed. The elution was carried out with 5 mL of methanol/formic acid (94:6 v/v) solution, the second elution was performed with 5 mL of methanol and the third was performed with 5 mL of methanol/NH_3 (95:5) solution. All solutions collected after elution of the cartridges were evaporated by a flux of N_2 at a temperature of about 30°C and the residue dissolved, in the first analyzes, with 1 mL of mobile phase and in the last analysis the dissolution was done with methanol/mobile phase (25:75 v/v).

1.1.2 HPLC analysis

To determine the suitable wavelength for the detection of each pharmaceutical, experiments were performed using high performance liquid chromatography coupled to the detection by diodes. Wavelength values between 220 and 300 nm were chosen, the range that the equipment allows to monitor, that presented well defined peaks with a larger area. The choice was made from a sweep considering the bottom line. Beckman Coulter (system gold) HPLC equipment was used with a diode detector (DAD) (module 168) and an automatic injector (module 508). The analytes were separated using a Kinitex 2.6 µm C18 100x4.6 mm column. The eluents used in the pharmaceuticals analysis were an eluent A (water/formic acid, 99:1 v/v) and an eluent B (acetonitrile). Eluent A was pre-filtered using a vacuum filtration system, then both eluents were degassed in an ultrasound equipment (Elma, Transsonic model 460 / H).

1.1.3 Results

Initially, the compounds were analyzed in the HPLC using various wavelengths. It was verified through their chromatograms that in the analysis of pharmaceuticals two different wavelengths should be selected, one of 298 nm for paroxetine and one of 252 nm for bezafibrate and simvastatin, to obtain well defined peaks and with significant areas.

Analyzes performed by HPLC also allowed to conclude that the quantification of the pharmaceuticals can be made based on calibration lines obtained using aqueous standard solutions (linearity between 0.2 and 8 mg/L).

For the SPE, two types of cartridges were used: Oasis HLB and Oasis MCX. It was concluded that the most suitable type for paroxetine and bezafibrate was HLB (recovery between 60 and 80% for acidic pH of 2) and for simvastatin is was MCX (recovery between 40 and 70%). However, MCX cartridges also showed to be suitable for paroxetine and bezafibrate analysis. The recovery percentages in the SPE procedure for paroxetine (elution methanol/NH_3 (95:5) solution) and bezafibrate (elution with methanol/formic acid (94:6 v/v) solution) were, respectively, 42 and 80% for aqueous standard solutions.

1.2 Development of methods of analysis of emerging pollutants (pharmaceuticals) in sediments

In the study of Antonio Francisco Cebrian Talaya (2015), an analytical method that was simple and quick to measure the anti-lipid compounds (simvastatin, bezafibrate and gemfibrozil) and antidepressants (paroxetine and fluoxetine) in marine sediments extracted from estuaries was develop. The methodology included extraction by ultrasound extraction (USE) (2 sequential extractions), extraction by vortex agitation (VA) and microwave assisted extraction (MAE), followed by a chromatographic analysis step in HPLC, also evaluating the need for a pre-

treatment of the extracts by SPE.

The three sediment extraction techniques were compared in terms of recovery efficiency of the drugs by selecting different extraction solvents (Table 1). The best technique was then selected, and the effect of the SPE application to eliminate the interferences in some cases due to the matrix effect was evaluated. HPLC with diode array detector (DAD) was used for the analyzes.

The SPE and HPLC analysis procedures were the ones optimized in the previous study described above.

Table A.1.1 - Sediment extraction techniques and solvents tested for pharmaceuticals extraction from sediments

Solvent (v:v)	**Volume of solvent**	**Mass of sample** (g)	**Process (time of extraction)**
Methanol/Formic acid (95:5)	5	1	USE (2 cicles of 15 min), VA (5 min) and MAE (20 min)
Methanol/Water (95:5)	5	1	USE (2 cicles of 15 min), VA (5 min) and MAE (20 min)
Methanol/NH_3 (95:5)	5	1	USE (2 cicles of 15 min), VA (5 min) and MAE (20 min)
Methanol	5	1	USE (2 cicles of 15 min), VA (5 min) and MAE (20 min)
Methanol/Acetone (95:5)	5	1	USE (2 cicles of 15 min), VA (5 min) and MAE (20 min)

The percentages of recovery of the pharmaceuticals (Figures A.1 and A.2) obtained for the extracts of the sediment samples processed using the different extraction techniques, were compared with the objective of obtaining the best conditions for drug extraction.

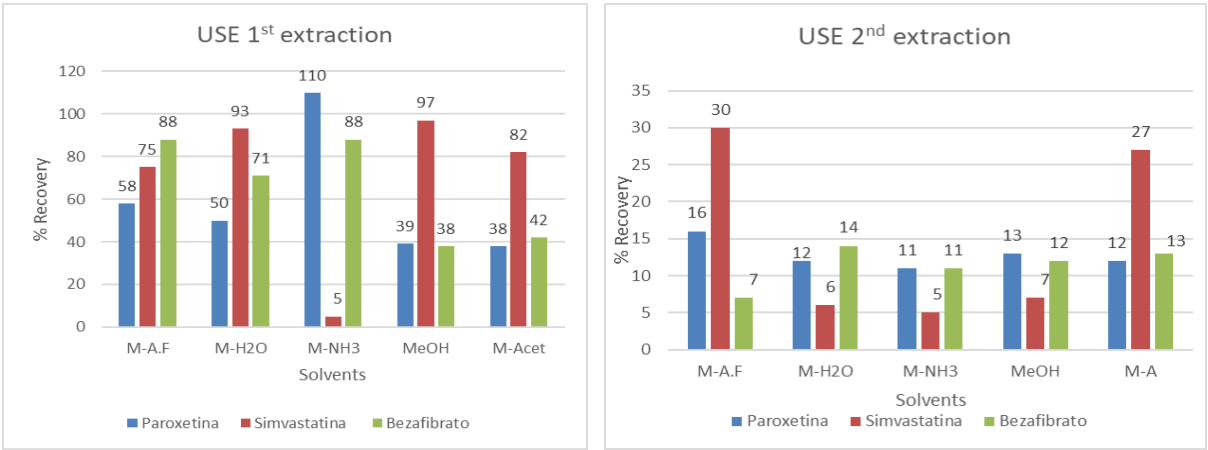

Figure A.1.1 – Figures from the study concerning the recoveries of the first and second extractions using ultrasound extraction.

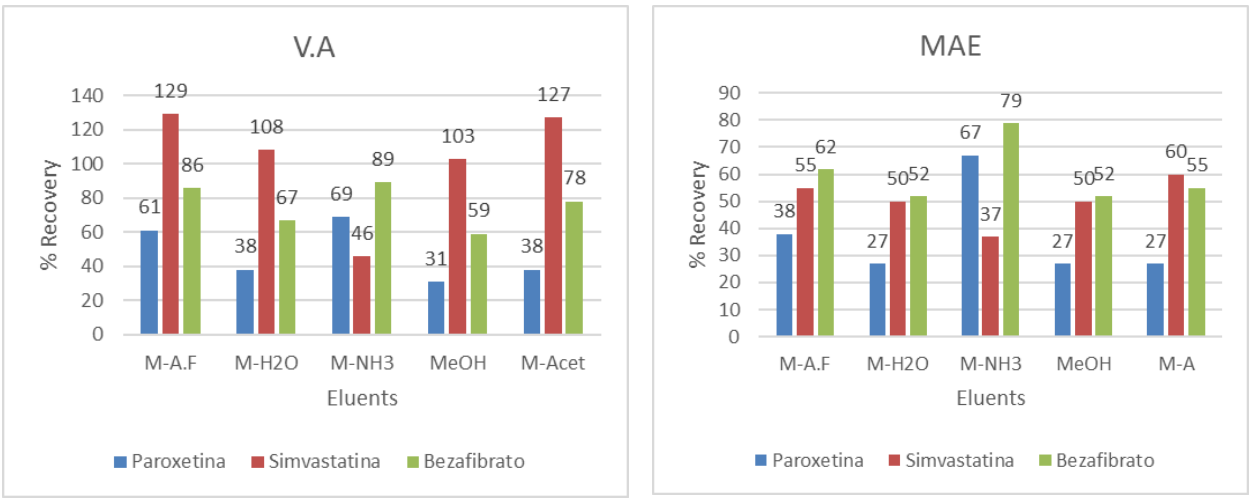

Figure A.1.2 – Figures from the study concerning the recoveries of each compound using extraction by vortex agitation (V.A) and microwave assisted extraction (MAE).

Attending for the techniques, it is possible to conclude that the best technique for the extraction of this type of drugs was USE with 2 sequential extractions because there were still significant amounts of drug adsorbed in the sediment after the first extraction.

In the case of solvents, to obtain good results for paroxetine the best solvent is M-NH3. For simvastatin the best solvent was M-F.A., followed by M-H2O. For bezafibrate the best solvents were M-NH3 and M-Acet.

Based on the fact that the USE technique with two extractions was the most appropriate, the same ultrasonic extraction procedure was done, followed by SPE to eliminate possible interferences of the matrix (Figure A.3).

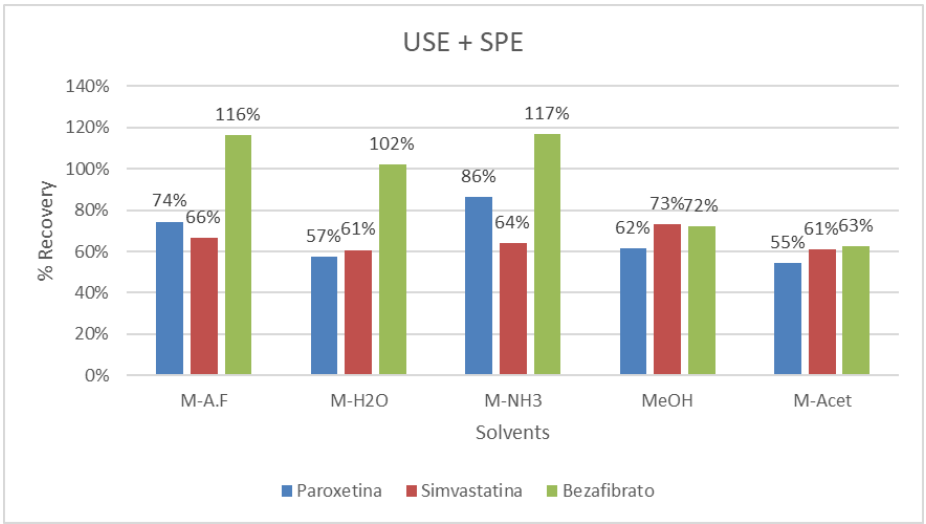

Figure A.1.3 – Figure from the study showing the recoveries of using ultrasound extraction along with solid-phase extraction.

Observing the results, it is possible to conclude that, under the experimental conditions used,

there are not many advantages when using SPE to eliminate the possible interferences of the matrix, being the recoveries obtained similar with and without SPE.

So, the methodology optimized to extract the selected pharmaceutical from sediment was two sequential extractions in an ultrasonic batch at room temperature with a solution of Methanol/NH_3 (95:5). The obtained extracts were evaporated until dryness by a flux of N_2 at a temperature of about 30°C and the residue dissolved with a methanol/mobile phase (25:75 v/v) solution. The HPLC analysis described above was then carried out.