Response of microorganisms from natural and constructed wetlands to veterinary drugs

Joana Pereira Fernandes

Mestrado em Ciências e Tecnologia do Ambiente

Departamento de Geociências, Ambiente e Ordenamento do Território.

2014

Orientador

Ana Paula de Campos Mucha, Investigadora Auxiliar, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Co- orientador

Maria Clara Ramalho Monteiro Pires Basto, Professora Auxiliar, Faculdade de Ciências da Universidade do Porto

Acknowledgment

In first place, I want to thank to Ana Paula Mucha, for the orientation, the availability, exigency, several work discussion, her help in all my doubts and mishaps along this thesis. To Professor Maria Clara Basto, for the availability and support. To Marisa Almeida, for her help, availability and support on the chemical area of this work. During this work I have grown professionally and without their support this would not be possible.

To European Regional Development Fund (ERDF) through COMPETE - Operational Competitiveness Program and national funds through FCT, under PEst-C/MAR/LA0015/2013, and ECORISK (reference NORTE-07-0124-FEDER-000054), co-financed by the North Portugal Regional Operational Programme (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF) for financing this project.

To CIIMAR - Interdisciplinary Centre of Marine and Environmental Research and Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade do Porto, for the use of the installations and facilities.

To Pedro Carvalho, for giving me the sediment samples from his PhD thesis which allowed me to perform the microbial experiments and also for reviewing chapter 2 of my master thesis.

To all my laboratory colleagues at CIIMAR and FCUP, for the great environment and support. Especially to Izabela Reis, for her precious help in the learning of all methodologies, her patient and dedication.

I could not forget my family, for all strength, patience, kindness and comprehension in this moment of my life. Without them, it would not be the person that I am today and none of this would be possible.

I also want thank to my boyfriend, Daniel Queirós, for all the support, understanding and above all, the strength that he has given me. His presence has made this phase much easier to overcome.

Last but not least, to Ana Matos, Margarida Silva, Tania Oliveira, Marta Macedo and Ana Cunha, for all support, friendship, strength and for all great and awesome moments.

Abstract

In the last years, contamination of freshwater systems by chemical compounds has been increasing along with human development, being many of these chemicals emerging environmental pollutants. These compounds can be a potential threat to public health and the environment but remain without regulations. Pharmaceuticals (including veterinary antibiotics), polar pesticides, veterinary products, among others, are examples of emerging pollutants.

Veterinary antibiotics have been widely used in livestock industry in an intensive and uncontrolled way leading to its detection in wastewater, freshwater and groundwater. Conventional methods of wastewater treatment are generally not capable or equipped to remove these compounds therefore, they are released without efficient treatment. Consequently, veterinary antibiotics or their active compounds can enter directly in the water system through effluent discharges. In addition, veterinary antibiotics can reach the environment indirectly through manure's lixiviation used as organic fertilizer. Despite of being found at low concentrations, they can cause toxic effects in organisms and promote antibiotic resistance.

Natural wetlands, like salt marshes present in estuarine areas, are characterized by the presence of water and adapted vegetation in saturated conditions and unique soils that differ from upland soils. Furthermore, wetland has a high rate of biological activity having the potential to transform several common pollutants, some presented in wastewater treatment plants, in harmless byproducts or essential nutrients that can be used for additional biological productivity.

Constructed wetlands (CWs) are being considered a potential technology to remove pharmaceuticals from wastewater effluents, but their ability to improve water quality depends greatly on their microbial communities. They are designed to mimic natural wetlands, being based on the interactions among soil/sediment, plant and microorganisms to remove contaminants from effluent.

The aim of this study was to understand the response of microbial communities from natural and constructed wetlands to veterinary antibiotics. For that, two different experiments were performed using the salt marsh plant *Phragmites australis,* which is commonly found in Portuguese estuarine areas and has been widely used for wastewater treatment in CW in America and Europe.

In one of the experiments, microbial community dynamics associated with veterinary antibiotics (enrofloxacin and tetracycline) removal from livestock wastewater was evaluated in CWs microcosms, in terms of abundance, diversity and community structure. Results point to CWs applicability for veterinary antibiotics removal from

livestock wastewaters, showing that CWs microbial communities were able to adapt without significant changes in their diversity or depuration capacity.

On the other experiment, the response of a salt marsh plant-microorganisms association to a contamination with a veterinary antibiotic (enrofloxacin) under different nutritional conditions was evaluated using natural estuarine water and sediments. Results showed that the presence of veterinary antibiotics in estuarine areas can affect their microbial community structure and that salt marsh plants and associated microorganism present a potential for antibiotic removal that is highly dependent on their nutritional status.

This study emphasizes the potential salt marsh plant-microorganisms association for the removal of veterinary antibiotics contamination from both natural and constructed wetlands, showing promising results for its application in the remediation of the environmental impact of these contaminants.

Keywords: Constructed wetlands; natural wetlands; veterinary antibiotics; plant-microorganisms association; bioremediation

Resumo

Nos últimos anos, com o desenvolvimento da população e das suas necessidades, tem sido registado um aumento da contaminação nas matrizes aquosas por compostos químicos, sendo muito destes compostos considerados poluentes emergentes. Estes compostos podem representar uma potencial ameaça para a saúde pública e para o próprio ambiente mas estes continuam sem regulamentação. Os produtos farmacêuticos, incluindo antibióticos veterinários, pesticidas polares, produtos veterinários, entre outros, são exemplos de poluentes emergentes.

Os antibióticos veterinários têm sido amplamente utilizados na pecuária de forma intensiva e não controlada levando a sua deteção nas águas residuais bem como nas águas superficiais e subterrâneas. Os métodos convencionais de tratamento de águas, no geral, não estão equipados para remover este tipo de compostos. Desta forma, os antibióticos veterinários ou os seus compostos ativos, entram diretamente nos sistemas aquosos através das descargas de águas residuais tratadas. Para além disso, os antibióticos veterinários podem alcançar o ambiente, de forma indireta, através da lixiviação de estrumes usados na agricultura como fertilizante orgânico. Os antibióticos têm sido detetados a baixas concentrações, contudo, estas concentrações podem causar efeitos tóxicos nos organismos e promover resistência a antibióticos.

As zonas húmidas naturais como, por exemplo, sapais presentes nas áreas estuarinas são caracterizadas pela presença de água e vegetação adaptada a condições de saturação e pelo tipo de solos único, que apresentam propriedades diferentes dos solos não vegetados. Além disso, as zonas húmidas naturais possuem uma elevada atividade biológica, tendo estas potencial para transformar alguns poluentes em subprodutos menos perigosos ou em nutrientes essenciais que podem ser utilizados para produtividade biológica adicional. Alguns dos contaminantes em questão, estão normalmente presentes nas águas residuais que chegam às estações de tratamento de águas residuais.

As zonas húmidas construídas, também conhecidas por leito de macrófitas, têm sido consideradas uma potencial tecnologia para remover fármacos das águas residuais. Contudo, a sua capacidade para melhorar a qualidade da água depende, em grande escala, das comunidades microbianas presentes. As zonas húmidas construídas são desenhadas de forma a imitar os processos que ocorrem nas zonas húmidas naturais, sendo estes baseados nas interações entre o solo/sedimento, planta e os microrganismos sendo assim possível remover os contaminantes do efluente.

O objetivo deste estudo foi compreender a resposta de comunidades microbianas em zonas húmidas naturais e construídas a antibióticos veterinários. Para

isso, foram realizadas duas experiencias diferentes usando uma planta de sapal, *Phragmites australis*, que é normalmente encontrada em áreas estuarinas Portuguesas. Esta planta tem sido amplamente utilizada no tratamento de águas residuais por zonas húmidas construídas na América e na Europa.

Numa das experiencias, foi avaliada, em zonas húmidas construídas, a dinâmica das comunidades microbianas associadas à remoção dos antibióticos veterinários (enrofloxacina e tetraciclina) das águas residuais de pecuária, em termos de abundância, diversidade e estrutura da comunidade. Os resultados obtidos apontam para a aplicabilidade das zonas húmidas construídas para a remoção dos antibióticos veterinários das águas residuais de pecuária, mostrando que as comunidades presentes nas zonas húmidas construídas foram capazes de se adaptar sem alterações significativas na sua diversidade ou capacidade de autodepuração.

Na outra experiência, foi avaliada a resposta da associação planta-microrganismo à contaminação por um antibiótico veterinário (enrofloxacina) sob diferentes condições nutricionais, usando água estuarina natural e solo estuarino natural. Os resultados obtidos mostraram que a presença do antibiótico veterinário nas áreas estuarinas pode afetar a estrutura da comunidade microbiana e que as plantas de sapal e microrganismos associados apresentam potencial para remover antibióticos, sendo este altamente dependente das condições nutricionais.

Este estudo destaca o potencial das associações entre planta – microrganismos de zonas de sapal na remoção de antibióticos veterinários em zonas húmidas naturais e zonas húmidas construídas, mostrando resultados promissores para a sua aplicação na remediação dos impactes ambientais provocados por este tipo de contaminantes.

Palavras- Chave: Zonas húmidas construídas; zonas húmidas naturais; antibióticos veterinários; associação planta – microrganismos; biorremediação.

Index

Figures Index

Tables Index

Abbreviations

ARISA – automated rRNA intergenic spacer analysis

ARISA AFLs – ARISA fragment lengths

BOD –biochemical oxygen demand

BSA – bovine serum albumin

COD – chemical oxygen demand

CWs – constructed wetlands

DAD – diode array detector

DAPI – 4',6'-diamidino-2-phenylindole

DDD – daily doses

DGAV – direção-geral de alimentação e veterinária

E – enrofloxacin treatment

ECDC – European centre for disease prevention and control

ECOs – emerging organic compounds

EN – enrofloxacin + nutrients treatment

ENC – enrofloxacin + nutrients + glucose treatment

ENR – enrofloxacin

HPLC – high-performance liquid chromatography

HSSF- CWs – horizontal subsurface flow constructed wetlands

Hybrid CWs – hybrid constructed wetlands

JRC – joint research center

K_a – acid dissociation constant

K_b – base dissociation constant

K_{ow} – octanol-water partition coefficient

LOD – limits of detection

MDS – multidimensional scaling

MW – molecular weight

N – nitrogen

OTUs – operational taxonomic units

P – phosphorus

PCR – polymerase chain reaction

PM – particulate matter

POP – persistent organic pollutants

S – unplanted systems

SF-CWs – surface free constructed wetlands

SP – planted systems

SPE – solid phase extraction

TAE – tris-acetate-EDTA

TCC – total cell count

TET – tetracycline

TSS – total suspended solids

VSSF – CWs – vertical subsurface flow constructed wetlands

WWTPs – wastewater treatment plants

Chapter 1

Introduction

1. Introduction

1.1. Background

Freshwater contamination by chemical compounds has been increasing along with human development being many of these chemicals emerging environmental pollutants. These are substances that are released into the environment at low but continuous rates, and for which no current regulations exists (Thomaidis et al., 2012; Rivera – Utrilla et al., 2013) even though they are considered a potential threat to public health and the environment. Production, use, and disposal of numerous chemicals that offer improvements in industry, agriculture, medical treatment, and even common household conveniences have carried out an increasing concern (Kolpin et al., 2002). Pharmaceuticals and personal care products, illicit drugs and drug of abuse, hormones and steroids, synthetic musks, bisphenol A, triclosan , triclocarban , as well as polar pesticides, veterinary products, industrial compounds/by-products, food additives and engineered nano-materials are examples of emerging pollutants (Lapworth et al., 2012; Thomaidis et al., 2012; Gavrilescu et al., 2014). They have reached the environment through anthropogenic sources (Gavrilescu et al., 2014) and have been detected in lakes, rivers, freshwater catchments, estuaries, reservoirs, raw/treated wastewaters and in marine waters. Emerging pollutants can be persistent in air, water, soil, sediments even at low concentrations (Gavrilescu et al., 2014).

In the last years, pharmaceuticals had a special attention as potential chemical contaminants in the environment (Rivera – Utrilla et al., 2013). The presence of pharmaceuticals went unnoticed for many years due to their occurrence in trace concentrations. Pharmaceuticals are compound biologically active that can affect non-target organism (Garcia – Rodríguez et al., 2014). Due to their characteristics, this type of pollutants requires some changes in the conventional approach of pollution prevention and control (Gavrilescu et al., 2014). Pharmaceuticals are divided in several therapeutic groups. The most usually detected are antibiotics, anti-inflammatory, analgesics, antidepressants, antiepileptics, lipid-lowering drugs, β-blockers, antiulcer drugs, antihistamines and other illicit drugs (heroin, methadone, etc.) (Rivera – Utrilla et al., 2013).

Pharmaceuticals are metabolized in very different ways. Their excreted metabolites and unaltered parent compounds can enter in water systems without significant changes due to biological, chemical and physical processes (Verllichi et al., 2012). Pharmaceuticals and some transformation products have been considerate pseudo-persistent compounds (Trovó et al., 2008; Thomaidis et al., 2012; Li, 2014).

Currently, Joint Research Centre (JRC) is developing “Watch List”, a pilot research exercise designed to anticipate and recognize future priority substances (JRC scientific and policy reports, 2012). This initiative, aims to put on surveillance several emerging pollutants known to represent a risk to surface water. For the first time, three pharmaceuticals (7alphaethinylestradiol, 17beta-estradiol and diclofenac) will also be included on a “watch list” of emerging pollutants which may, in the future, be included in the priority list (Euroactiv, 2013).

1.2. Antibiotics

Antibiotics are considered emerging environmental pollutants and they have been found in the environment. They are natural and chemical substances used to prevent or treat bacterial diseases. They are separated in two major groups: bacteriostatic antibiotics that are capable to suppress the bacterial growth and bactericidal antibiotics that are capable to kill bacteria. They are widely used in human and veterinary medicine being the fluoroquinolones, macrolides and aminoglycosides the most frequently prescribed antibiotics in human medicine and penicillins, tetracyclines and macrolides the most regularly prescribed in veterinary medicine (Milić et al., 2013). Antibiotics are produced to have a low biodegradability and high water solubility (Zhou et al., 2009).

Antibiotics can be released into the environment through point or diffuse sources. Point sources are much easy to identify comparing with diffusion sources. Pointed sources are located in separate locations and can be calculated by mathematical modelling (Li, 2014). The main point sources are sewage treatment plants, industrial effluent and hospital effluent (Li, 2014). The diffused sources can occur over board geographical scales and are very difficult to identify. Agricultural runoff from the animal waste and manure, urban runoff from domestic waste and the leakage from wastewater treatment plants are considerate diffused sources (Li, 2014).

1.2.1. Veterinary antibiotics

The use of antibiotics in the livestock industries has increased over the past few years to protect from or cure various diseases. In several parts of the world like Europe, US, UK, have been noticed the presence of numerous classes of antibiotics in the water matrixes being some of them known to be environmentally persistent (Zhang et al., 2014). There are innumerous antibiotics in the environment and their prioritizing is needed. In Portugal, enrofloxacin (ENR) and tetracycline (TET) are two of the veterinary antibiotics that are highly consumed (Carvalho, 2012).

Present research was focused in two veterinary drugs, enrofloxacin and tetracycline (fig. 1).

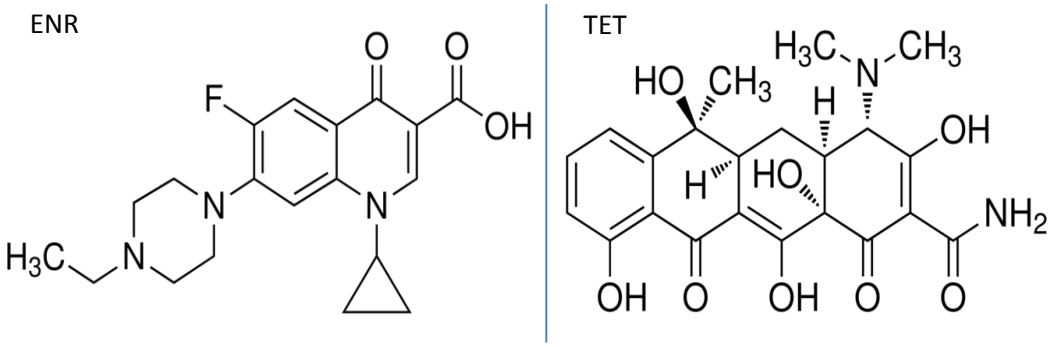


Fig. 1 - Enrofloxacin (ENR) and tetracycline (TET) structure.

Enrofloxacin belongs to fluoroquinolones family, a class of synthetic antibacterial that has broad-spectrum antibiotic properties (Knapp et al., 2005). ENR has a high environmental interest not only because their intensive use in livestock industry but also because one of its primary degradation products is ciprofloxacin (Knapp et al., 2005), another antibiotic that is released in the environment.. ENR is photodegradable with half-lives of 5 minutes to 5 hours. This phenomenon depends on light intensity, pH, phosphorous level and presence of organic particles. ENR also has the ability to adsorb into organic matter (Knapp et al., 2005 and references therein).

Tetracycline belongs to tetracycline's family which are broad-spectrum agent, showing activity against a varied range of gram-positive and gram-negative bacteria, atypical organisms and protozoan parasites (Chopra & Roberts, 2001). They are commonly used in pig's creation (Qiao et al., 2012). Adsorption into activated sludge with no biodegradation was reported by Li & Zhang (2010).

Physical/chemical properties of ENR and TET are represented in table 1.

Table 1 – Important physical/chemical properties of enrofloxacin and tetracycline (base on Sarmah et al., 2006).

Antibiotics	pKa (25ºC)	pKb (25ºC)	Solubility	Vapour pressure (Torr)	Henry's law constant (Pa m^3 mol^{-1})	Log K_{ow}	MW (g mol^{-1})
Enrofloxacin	2.74	7.11	130000	2.10×10^{-13}	5.2×10^{-17} – 3.2×10^{-8}	2.53	359.4
Tetracycline	3.3–9.6	n.a	1700	n.a	---------- ---------	------- ---	444.4

n.a – not available

1.2.2.Occurrence of veterinary pharmaceuticals in the environment

Livestock industry has been increased along the years to satisfy the human's needs. Therefore, the use of veterinary pharmaceuticals to treat diseases as well as growth promoters has increased. About 75% of the antibiotics are excreted as active metabolites being this the major source of antibiotic input into the environment (Pei et al., 2007).Consequently, veterinary antibiotics or their active compounds can enter directly in the water system through effluent discharges. In addition, veterinary antibiotics can reach the environment indirectly through manure's lixiviation used as organic fertilizer produced in agriculture (Carvalho et al., 2014) (fig. 2).

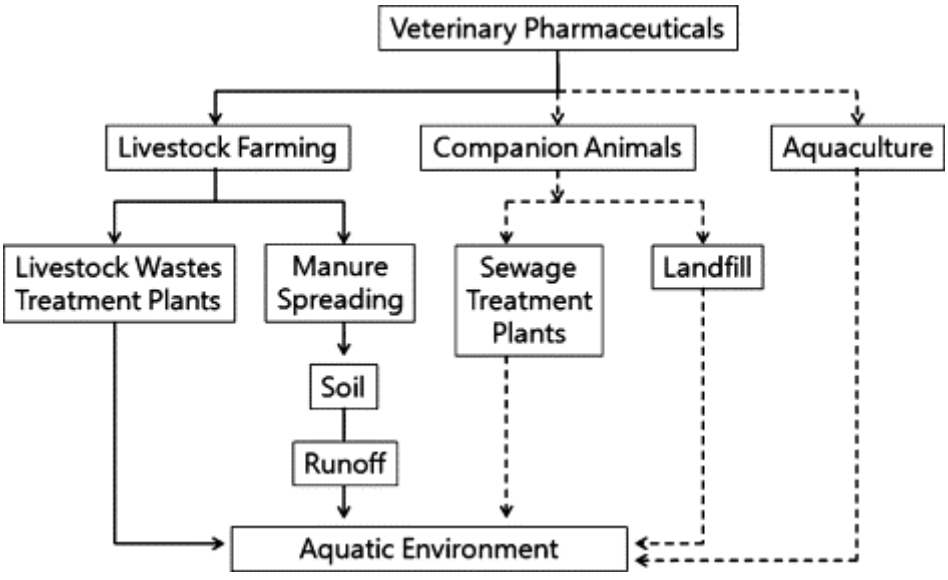


Fig. 2 - Veterinary pharmaceuticals inputs in aquatic environment (Kim et al., 2008).

Conventional methods of wastewater treatment are generally not capable or equipped to remove these compounds therefore, they are released without efficient treatment (Kim & Aga 2007). The most part of wastewater treatments plants (WWTP's) have a primary physic-chemical treatment based on harrowing and flocculation processes and secondary biological treatment with biological reactors being most of them active sludge reactors. The major problem of this conventional biological treatments lies on lack of capacity of microorganisms to metabolize these compounds because most of them cannot be metabolized by microorganisms as source of carbon (Rivera – Utrilla et al., 2013). In addition, antibiotics can inhibit the microorganism's activity (Rivera – Utrilla et al., 2013).

On the other hand, some antibiotics can be removed in primary treatment by adsorption to the major suspended particles that are removed by harrowing and flocculation processes; however, the conventional methods are incapable to effectively remove most of antibiotics from wastewater. Furthermore, antibiotic´s physical and chemical properties, specifically adsorbability, absorbability, biodegradability, solubility, volatility, polarity and stability vary greatly, with clear impacts on their behavior during the treatments and, in this way, in their removal efficiencies (Verllichi et al., 2012).

Other technologies do exist such as advanced oxidation processes (photo-Fenton, Fenton-oxidation), chlorination, adsorption by activated carbon or membrane processes (reverse osmosis ultra-filtration and nano – filtration), but they entail cost effectiveness (Metcalf & Eddy, 2003; Zhang et al., 2014).

1.2.3.Environmental effects caused by antibiotics

Even though antibiotics are found at low concentrations in the environment (ng L^{-1} to µg L^{-1}) (Li & Zang, 2010), they can cause serious toxic effects in organisms and promote antibiotic resistance (Halling-Sørensen et al., 1998); Zhang et al., 2014).

Several authors reported bacteria resistant in the aquatic environment and in soil (Pei et al., 2007; Kümmerer, 2009b; Fatta – Kassinos et al., 2011). The use of antibiotics as growth promoters have been debated once they can cause a selective pressure for bacteria that are resistant to antibiotics compromising their continued use (Kümmerer, 2009a).

Microbial communities as well as bacterial diversity present in the environment are susceptible to antibiotics effects (Hammesfahr et al., 2011; Ollivier et al., 2013). In fact, in general antibiotics have selective effects on various groups of microbes, even those designed to be broad-spectrum drugs (Ding & He, 2010). Effects of antibiotics in microbial community depends on microbial groups present, antibiotics concentration,

original soil properties (Ding & He, 2010 and references therein) and animal species and its respective nutrition and gut (Jechalke et al., 2014). Thiele-Bruhn & Beck (2005), reported selected pressure on soil microbial community by antibiotics at trace concentrations.

In addition, antibiotics have a potential as endocrine disruptors (Jones et al., 2001; Fent et al., 2006) and can cause reproduction inhibition (Park et al., 2007). Moreover, high concentrations of antibiotics in the wastewater can affect the biological wastewater treatment in terms of their stability and performance due to the resilient bacteriostatic effects of antibiotics and can cause changes in the microbial community present in the biological treatment (Deng et al., 2011). Furthermore, the continuous input of these compounds in the environment can originate some toxic mixtures that can induce unnoticed adverse effects on aquatic and terrestrial organisms (Wille et al., 2010) as well potential ecosystem-level responses involving non-target species (Kim et al., 2008 and referenced therein).

1.3. Antibiotics consumption in an European perspective

1.3.1. Tetracyclines

Surveillance of antimicrobial consumption in Europe is a report where information about antibiotics consumption data from the community is available (ECDC, 2011).

In 2011, tetracycline consumption was generally lower in southern Europe than in northern and western Europe (fig. 3). Consumption of tetracyclines in Portugal ranged between 0.3 to 1.08 DDD (daily doses) per 1000 inhabitants and per day, having the lower consumption registered in 2011.

Doxycycline was the most consumed in the community in 2011 followed by lymecycline, minocycline and tetracycline.

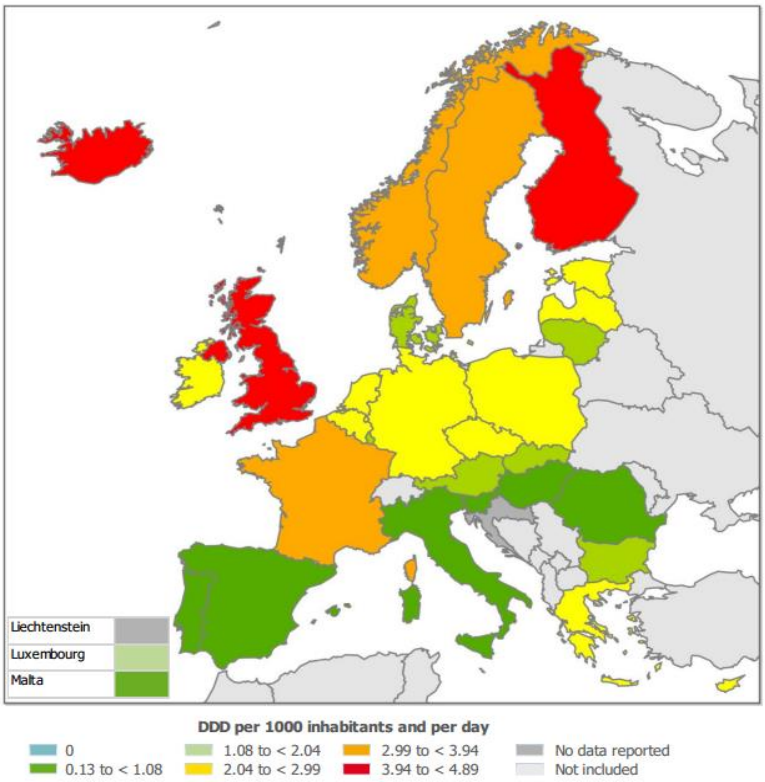


Fig. 3 - Consumption of tetracyclines in EU/EEA countries, in 2011 (ECDC, 2011).

1.3.2. Quinolones

In fig. 4 is represented the consumption of quinolones (first, second and third generation) in surveillance report of 2011(ECDC, 2011).

Quinolones consumption was as generally lower in northern Europe and western Europe comparing with south Europe (fig. 4). Consumption of quinolones in Portugal ranged between 2.45 to 3.12 DDD per 1000 inhabitants and per day having a high consumption comparing with other countries.

Fluoroquinolones, mainly ciprofloxacin, was the most consumed, contributing for almost entire consumption of quinolone antibacterial.

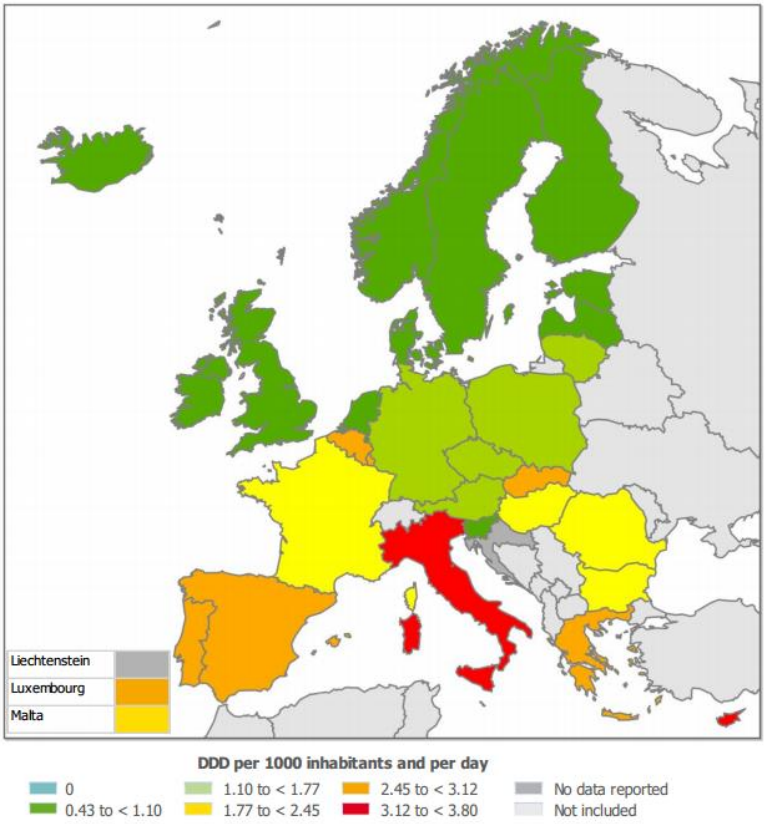


Fig. 4 - Consumption of quinolone antibacterials in EU/EEA countries, in 2011 (ECDC, 2011).

1.4. Legislation

The legislation applied in veterinary medicine in Portugal is available in DGAV site (Direção Nacional de Alimentação e Veterinária). DL 237/2099, September 15th; DL 314/2009, October 28th are available in this platform as well the list of authorized veterinary products and revoked veterinary products. However, no information was found specifically for enrofloxacin and tetracycline in terms of recommended and limited doses in livestock industry.

1.5. Wetlands

1.5.1.Natural wetlands

Natural wetlands are a natural resource during human history. They are wet areas during part or all of the year due to their location in the landscape (fig. 5) (Kadlec & Wallace, 2008) and they have natural deputation ability that has been recognized as an attractive option in wastewater treatment (Scholz & Lee, 2005). Besides, wetlands can be seen as natural recreational areas for local community (Scholz & Lee, 2005) having some social and economic value.

Fig. 5 - Natural wetlands
(A - http://upload.wikimedia.org/wikipedia/commons/thumb/6/6a/Wetlands_(Moscow,_Russia).jpg/800px-Wetlands_(Moscow,_Russia).jpg ; B - http://www.theepochtimes.com/n2/images/stories/large/2009/09/02/NYClub90175275.jpg).

The main characteristics of wetlands are the presence of water, the presence of vegetation adapted to saturated conditions and unique soils that differ from upland soils (Scholz & Lee, 2005 and references therein). These characteristics make wetlands one of the most biologically productive ecosystems in the planet. Moreover, wetland has a high rate of biological activity comparing with other ecosystems and they have the potential to transform several common pollutants, some released by wastewater treatment plants, in harmless byproducts or essential nutrients that can be used for additional biological productivity (Kadlec & Wallace, 2008).

1.5.2.Estuaries

Most estuarine areas include natural wetlands ecosystems. Estuaries can support a range of different wetlands habitats, taking into account their specific characteristics (Dugan, 1990). In temperate estuaries, salt marshes, intertidal mud and sand flats are the most common features (Dugan, 1990).

Estuaries are semi – enclosed and tidal coastal bodies of water which has a mixture of freshwater from the rivers and coastal stream merges from the sea (Chapman & Wang, 2001; Gillanders et al., 2011; Sun et al., 2012). Estuaries (fig. 6 A and B) are different from other ecosystems since they have a mixing of waters with different types of salt concentrations. This fact allows them to have unique physical conditions that support extremely diverse organisms and offer essential relations to near ecosystems (Sun et al., 2012).

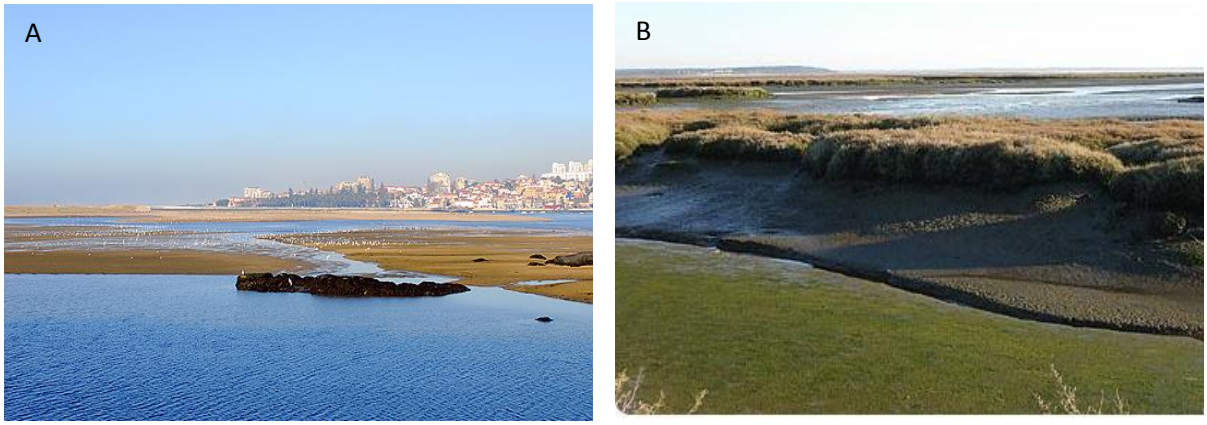


Fig. 6 - Estuarine areas: A – Douro River Estuary, North of Portugal (http://www.avesdeportugal.info/images/Estuario_Douro_1.jpg); B – Sado River Estuary, South of Portugal (http://blog.toprural.pt/wp-content/uploads/2012/08/512px-Estu%C3%A1rio_do_Sado_3.jpg).

The interface among fresh and salt water provides strong gradients in many physical and chemical variables including salinity, pH, temperature, nutrients, solved oxygen and redox potential (Chapman & Wang, 2001).

Estuaries are highly productive ecosystems (Gillanders et al., 2011; Jones et al., 2011) that have a crucial role in biogeochemical cycles (Jones et al., 2011); however, they are one of the most sensitive and fragile ecosystems (Bouvy et al., 2010) and, as a result, very difficult to recover. In addition, several estuaries are suffering of eutrophication and losing water quality due to high nutrients loads and pollutant inputs (Bouvy et al., 2010).

Several contaminants like, pesticides, pharmaceuticals, personal care products and other industrial compounds have been detected in estuarine areas. The continuous input of chemical contaminants into estuarine areas through rivers, lagoons, wastewater treatment plants outfalls (Klosterhaus et al., 2013) and illegal discharges can cause serious effects in several organisms, ecosystem degradation, habitats deterioration and possible human poisoning (Pan & Wang, 2012 and references therein).

Microbial communities from estuaries have a very important role in the ecosystem activity; however, they are exposed to several environmental changes. The organic and inorganic compounds dissolved or suspended in the water, the mixing of freshwater and

seawater as well as hydrological variations due to precipitation can cause specific patterns of microbial abundance, diversity and activity in estuaries (Bouvy et al., 2010 and references therein). Due to the mixing of freshwater and seawater, estuaries have a variable salinity gradient. Salinity is considerate a stressful factor for microbial communities from freshwater and have a significant effect in their functioning and performance (Lozupone & Knight, 2007).

Estuarine sediment was defined by Chapman & Wang (2001) as "sediments whose interstitial salinities are neither truly fresh nor truly saline; that is, they range above 1 and below 30 g L^{-1}". Salinity gradient in estuarine sediments is a very important factor once salinity shifts can affect the availability of contaminants therein (Chapman & Wang 2001). Salinity also affects partitioning of contaminants between sediments and overlying or interstitial waters affecting the bioavailability of contaminants in estuarine sediments. Contaminant bioavailability is measured by the reactivity of each contaminant with the biological interface (Eggleton & Thomas, 2004).

Salt marshes plants are essential in the maintenance of estuarine ecosystems. They are responsible for nutrients pools in sediments, elimination of stored nutrients such as phosphorus from soil and releasing them above the surface through tissue leaching (Weis et al., 2002). Brusati & Grosholz (2006) described that plants have an important role in water flow regulation, buffer salinity and sediment deposition.

The use of salt marsh plants for pollution control has been reported (ex: Almeida et al., 2011, Ribeiro et al., 2011). Salt marsh plants such as *Phragmites australis* (*P. australis*) and *Juncus maritimus* have been shown to have potential to be used in phytoremediation processes in estuaries to treat hydrocarbon and metal contamination (Nunes da Silva et al., 2014; Ribeiro et al., 2014). These results can lead to the hypothesis that salt marsh plants may also have a role in the emergent pollutants control, including pharmaceuticals, in estuarine areas.

1.5.3. Constructed wetlands

Constructed wetlands are engineered systems designed and constructed to mimic biological, chemical, and physical processes occurring in natural wetlands (fig. 7) (Zhang et al., 2014b). These processes include sorption, sedimentation, photolysis, hydrolysis, volatilization, plant uptake and accumulation, plant exudation, microbial degradation, filtration, precipitation and adsorption to remove pollutants from contaminated water within a more controlled environment (Garcia-Rodríguez et al., 2014; Wu et al., 2014). Enumerated processes can be directly and/or indirectly influenced by several factors like temperature, different loading rates, soil types,

operation strategies and redox conditions in the wetland bed (Wu et al., 2014). Constructed wetlands are ecological wastewater treatment that represents advanced and emerging solutions for environmental protection and restoration (Zhang et al., 2014b).

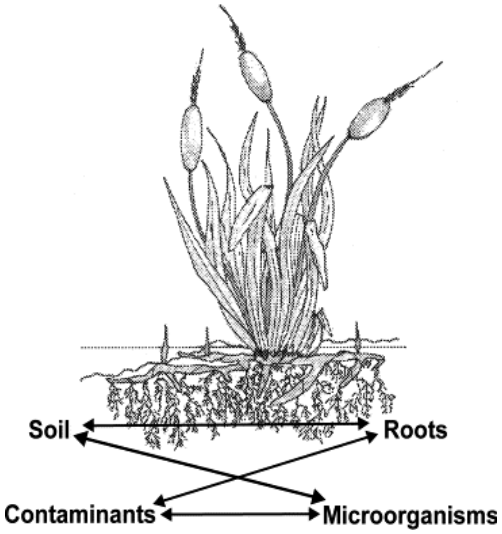


Fig. 7 - Possible interactions between plant, soil microorganisms and contaminant in constructed wetlands (Stottmeister et al., 2003).

This technology was, in first place, designed to treat domestic and municipal wastewater. Nonetheless, this technology was extended to animal and industrial wastewaters, agricultural effluents, urban and agricultural stormwaters, mine waters, landfill leachates, urban and highway runoff and groundwater remediation (Kadlec & Wallace, 2008; Wu et al., 2014 and references therein).

Constructed wetland have been widely used and have been recognized as potential alternative for WWTP's, since they have associated low cost and low energy requirements, easy operation and maintenance, high removal efficiencies of several contaminants, high rates of water recycling and potential for providing significant wildlife habitat (Zhang et al., 2014b and references therein).

Swamp plants like *Phragmites* sp. And *Typha* sp. Are usually used in constructed wetlands in Europe and Northern America (Kadlec & Wallace, 2008).

There are four types of constructed wetlands (fig. 8): surface free constructed wetlands (SF-CWs), horizontal subsurface flow constructed wetlands (HSSF- CWs), vertical subsurface flow constructed wetlands (VSSF – CWs) and hybrid constructed wetlands (hybrid CWs) (Li et al., 2014). They differed of each other in terms of layout, media, plants, and flow patterns (Kadlec & Wallace, 2008).

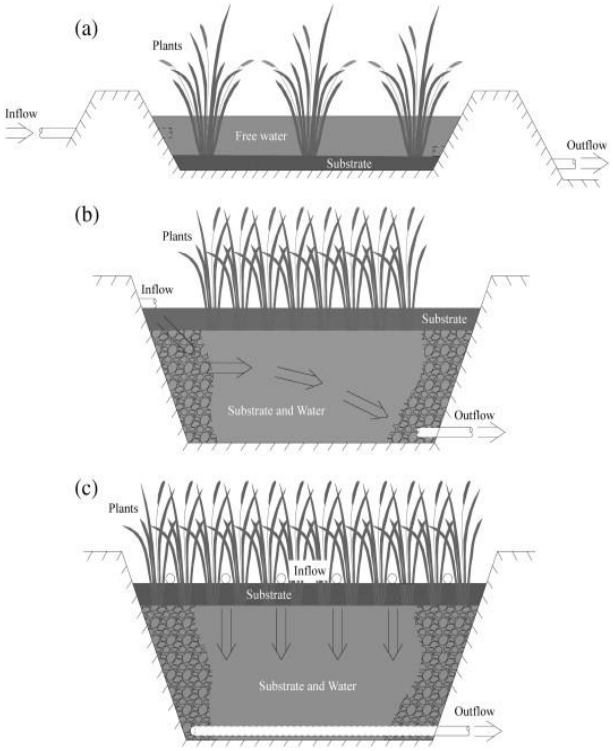


Fig. 8 - Different types of constructed wetlands (CW). A: SF – CW; B: HSSF – CW; C: VSSF – CW. (Li et al., 2014).

Surface free constructed wetlands are made of basins planted with vegetation (including rooted and floating plants) wherein the free wastewater flows at low depth over the impermeable bottom liner or the packed substrate layer (Li et al., 2014). The main treatment mechanisms are sedimentation, filtration, oxidation, reduction, adsorption, and precipitation (Kadlec & Wallace, 2008).

Horizontal subsurface flow constructed wetlands are made of gravel or soil beds planted with vegetation (Kadlec & Wallace, 2008). The wastewater (fed into the CW) flows horizontally through the substrate under the surface of wetland bed which is planted with vegetation. At the end of the treatment, the effluent is collected at the outlet zone (Li et al., 2014). This type of CW is typically used for primary treatment (Kadlec & Wallace, 2008).

In vertical subsurface flow constructed wetlands, the wastewater vertically flows crossing the planted layer down and the substrate until it reaches the outlet zone (Li et al., 2014). An advantage of this technology is the ability to treat concentrated wastewaters. In addition, this system can be combined with the other types of CW described above to create nitrification-denitrification treatment (Kadlec & Wallace, 2008).

The combination of two or more types of constructed wetlands is designated as hybrid CW (fig. 9).

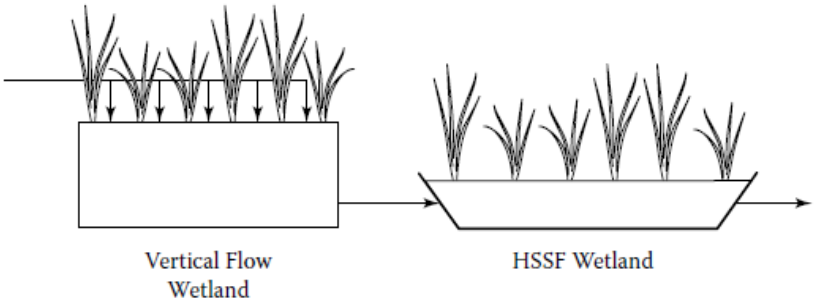


Fig. 9 - Hybrid constructed wetland (Kadlec & Wallace, 2008).

1.5.3.1. Importance of plant and microorganism

Removal efficiencies of constructed wetlands highly depend on plant/microorganisms interactions. These interactions allow the removal of contaminants from wastewater/water based on the increase of microbial population numbers in the rhizosphere (Oliveira et al., 2014). Plant has a strong influence in soil microbial communities. Microbial communities are stimulated by plant root exudates rich in carbon, nutrients and enzymes (Bais et al., 2006; Salvato et al., 2012; Oliveira et al., 2014) allowing the contaminant degradation. However, plant exudates and excreted exogenous enzymes can affect microbial community composition and diversity and, consequently, affect enzyme activity (Salvato et al., 2012 and references therein). Fester et al. (2014) described that "plant associated microorganisms often seem to be the real players mediating the plant impact on contaminant transformation".

Plants also give a strong mechanical stability to wetlands in the presence of contaminants (Fester et al., 2014).

1.5.3.2. Main mechanisms of antibiotics removal in CW

The main mechanisms of contaminant removal that can occur in CW's are represented in fig. 10 and they are described in detail in the next topics.

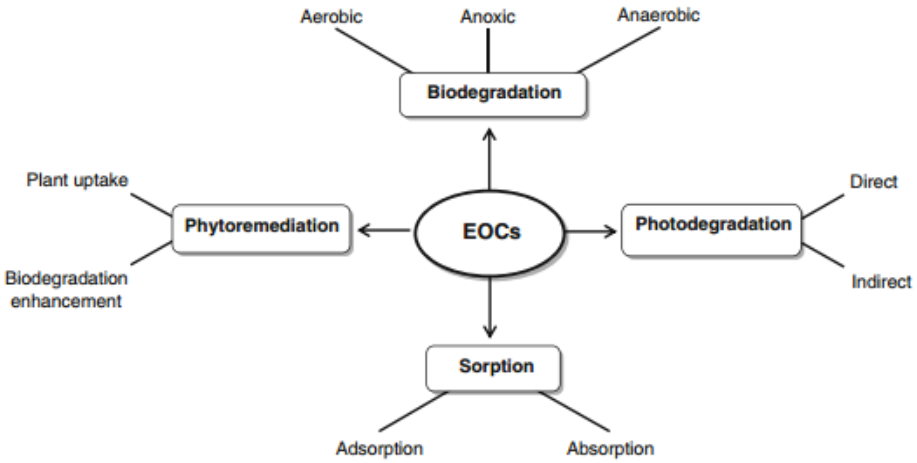


Fig. 10 - Main removal mechanisms for emerging organic compounds (ECOs) in CW's (Garcia – Rodríguez et al., 2014).

1.5.3.2.1. Sorption

Sorption into organic matter (suspended and unsuspended) is one of the CWs mechanisms to remove antibiotics from wastewater. Antibiotics can strongly adsorb to organic particles or sediment. To evaluate sorption behaviour, sorption coefficient is used to model sorption contaminants in sediment and soils. Antibiotics highly hydrophobic have a high potential to adsorb in CW substrate (Garcia – Rodríguez et al., 2014). In addition, hydrophobic compounds can highly adsorb into organic matter present in the granular medium (Garcia et al., 2010) and, because of that, becomes more recalcitrant to biodegradation resulting in high accumulation on the medium in wetlands (Zhang et al., 2014).

Sorption behaviour of antibiotics also depends on the chemical structure of the compounds and because of that, adsorption can occur due to electrostatic interactions of positively charged groups of chemicals with negatively charged surfaces (Zhang et al., 2014)

1.5.3.2.2. Photolic degradation

Photodegradation is one of the most predominant processes in the degradation of antibiotics from wastewater in CW's (Zhang et al., 2014). This process depends on sunlight availability (Garcia – Rodríguez et al., 2014), light intensity and light attenuation by water depth (Jechalke et al., 2014; Zhang et al., 2014). Photodegradation process can occur in direct way, which is through absorption of solar light by aquatic pollutants followed by chemical reactions or through indirect way in, which contaminants are

degraded by strong oxidant species such as hydroxyl radicals created by natural photosensitizers (Garcia – Rodríguez et al., 2014 and references therein).

1.5.3.2.3. Plant uptake and phytodegradation

Remediation processes involving plants and algae are used for clean – up aquatic media (surface and ground water) soils and sediments (Garcia – Rodríguez et al., 2014). These remediation processes of organic pollutants can occur by plant uptake, plant exudates and enzymes or with microorganism's participations (Garcia – Rodríguez et al., 2014).

The uptake potential of organic compounds is estimate by log K_{ow}. Some studies indicate high removal efficiencies for compounds with log K_{ow} values ranging from 0.5 to 3 (Zhang et al., 2014 and references therein). Salt et al., 1998, reported that organic pollutants may achieve partial or full degradation or they may be metabolized or transformed in to less toxic compounds. However, the toxicity of intermediates produced by plant remains an issue and has to be taken into account (Zhang et al., 2014).

1.5.3.2.4. Microbial degradation

Few studies have reported about biodegradation of pharmaceuticals in CW's. During biodegradation can occur mineralization or transformation into more hydrophobic or more hydrophilic compounds which remain in the liquid phase (Garcia – Rodríguez et al., 2014; Zhang et al., 2014). This process can occur in CW's under aerobic and anaerobic conditions being the aerobic degradation faster than anaerobic degradation (Garcia – Rodríguez et al., 2014).

However, it is unlikely that antibiotics present in wastewater can be effectively degraded by biodegradation alone. The low concentrations of antibiotics, comparing with other pollutants present in wastewater, may be insufficient to induce enzymatic degradation processes (Zhang et al., 2014). Other factor involved is the bioactivity of antibiotics, which can inhibit growth or metabolism of microorganisms (Zhang et al., 2014).

1.6. Aim of master thesis

The aim of this master thesis was to understand the response of microbial communities from natural and constructed wetlands to veterinary antibiotics. For that, two different experiments were performed:

Constructed wetlands study: Microcosm's experiments in greenhouse conditions to evaluate changes in microbial community structure caused by the presence of two veterinary drugs, enrofloxacin and tetracycline as well alterations in bacterial richness, diversity and microbial abundance.

Natural wetlands study: microcosm's experiments in laboratory conditions to evaluate the removal efficiency of ENR and assess the microbial community dynamics in terms of microbial structure, bacterial richness, diversity and microbial abundance.

The selected plant for this work was *P.australis* (fig. 11) which is commonly found in Portuguese estuarine areas. This plant can grow well in chemically reduced environments and water- logged soils (Armstrong & Armstrong, 1988). P. australis has been widely used for CW wastewater treatment in America, Australia and in Europe (Armstrong & Armstrong, 1988; Kadlec & Wallace, 2008).

Fig. 11 - *Phragmites australis.*

This master thesis is structured in 4 chapters. In chapter I, a general introduction is provided on antibiotics input and effects in the environment, natural and constructed wetlands and the major removal mechanisms that occur in wetlands.

Chapter 2 presents constructed wetland experiment. In this chapter, a brief introduction about constructed wetlands, material and methods applied, results and respective discussion and the major conclusion about the study is presented.

In chapter 3 is provide the natural wetland experiment. A short introduction about estuaries is provided followed by material and methods applied, results, discussion and main conclusions.

Finally, in chapter 4 are presented a general discussion of the work, where the results of both works are compared and debated, and final conclusions are presented.

Chapter 2

Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms

2. Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms

2.1. Introduction

Since conventional methods of wastewater treatment are not capable to remove antibiotics from wastewater, alternatives are need. A potential and sustainable alternative to remove antibiotics from wastewaters is constructed wetlands (CWs) (fig. 12). This technology can be used as a secondary or tertiary treatment and is designed to mimic natural wetlands, being based on the interactions among soil/sediment, plant and microorganisms to remove contaminants from effluents (Brix, 1994; Kivaisi, 2001). CWs can also be a way of managing water quality because it removes other compounds from wastewater besides antibiotics. Advantages of this technology are low costs, easiness of operation and maintenance, high quality effluent with less energy dissipation and strong potential for application in developing countries, particularly in small rural communities (Kivaisi, 2001; Carvalho, et al., 2012; Helt et al., 2012). However, this technology viability requires ample understanding of mechanisms removal, toxicity risks, environmental factors influence, removal efficiencies and design impacts (Li et al., 2014).

Fig. 12 - Constructed wetland in rural areas (http://images.sciencedaily.com/2013/09/130917124819-large.jpg).

These planted systems rely on the simultaneous occurrence of several complexes physical, chemical and biological processes, including sorption and sedimentation, photolysis, hydrolysis, volatilization, plant uptake and accumulation, plant exudation and microbial degradation (Garcia-Rodríguez et al., 2014).

Constructed wetlands efficiency for removal of conventional parameters like biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and nutrients from different wastewaters, including livestock industry

wastewaters, was already reported (Meers et al., 2008). In addition, application of CWs for pharmaceutical compounds removal from urban wastewaters has also been widely reported (e.g. Garcia-Rodríguez et al., 2014; Li et al., 2014; Verlicchi & Zambello, 2014). However, pharmaceuticals removal from livestock industry wastewaters has been only recently reported in very few works (Xian et al., 2010; Hussain et al., 2012, Carvalho et al., 2013). These effluents normally have much higher organic contents than those from domestic wastewaters, which makes them more difficult to treat.

Microbial communities present in CWs have an important role in water quality improvement. Several biological processes occur in CWs like, for instance, ammonia oxidation, denitrification and nitrogen fixation, which are mediated through different types of bacteria. Antibiotic presence, which can occur in livestock effluents, can affect depuration properties of CWs as well their functionality (Berglund et al., 2014). So, evaluating if antibiotics can affect CWs' microbial communities is necessary to fully validate this technology application.

This research purpose was to study the response of the microbial community from CWs microcosms used in a parallel study (Carvalho et al., 2013) to evaluate removal of two veterinary drugs (enrofloxacin (ENR) and tetracycline (TET)) from livestock industry wastewater. These compounds belong to two different antibiotic families: fluoroquinolones (ENR) and tetracyclines (TET). They were chosen due to their use in therapeutic in Portuguese livestock industry.

2.2. Material and methods

This work was a complementary study to an experiment developed by Pedro Carvalho in his PhD thesis (Carvalho, 2012). Therefore, sampling and microcosm's assembly (2.2.1), samples collection (2.2.2), antibiotic analysis (2.2.3) and toxicity test (2.2.4) were developed within that PhD thesis. All others procedures, namely, microbial abundance (2.2.5), DNA extraction (2.2.6), microbial community structure (2.2.7), electrophoresis agarose gel (2.2.8), PCR products purification and quantification (2.2.9 and 2.2.10) and statistical analysis (2.2.11), were developed within the present master thesis.

2.2.1.Sampling and microcosm's assembly

Plants (*P. australis*) with sediment attached to their roots (rhizosediment) were collected in Lima River (North of Portugal) in April 2012 (fig. 13).

Fig. 13 - Sampling site (Lima River Estuary, north of Portugal).

Sand was collected simultaneously in the river basin (within 1 m of plant stands). In the laboratory, sediment was separated from plant roots and mixed thoroughly with sand (1:1 proportion) to prepare roots' bed substrate for CWs microcosms. A small portion of the rhizosediment was maintained at -20ºC for posterior microbial community analysis (initial characterization).

Wastewater (after being treated in two anaerobic/aerobic lagoons) was collected every week in a pig farm, having on average pH of 8.04, COD of 1042 mg L^{-1} and 340 mg L^{-1} of particulate matter (PM), being 82 % organic PM (Carvalho et al., 2013).

Microcosms were set up in plastic containers (0.4 m x 0.3 m x 0.3 m) with 4 cm layer of gravel, 2 cm layer of lava rock and 10 cm layer of roots' bed substrate (fig. 14).

Half of the microcosms were planted with *P. australis*, whereas the others were left unplanted. Each system was wrapped in aluminum foil to avoid light penetration into the substrate, simulating a real system. Microcosms were designed to operate in batch mode having only a tap at the plastic containers base for sample collection.

Fig. 14 - Constructed wetlands – microcosms setup (Carvalho, 2012).

Three treatments were tested: one only with wastewater (control), one with wastewater doped with 100 µg L^{-1} of ENR and another with wastewater doped with 100 µg L^{-1} of TET. This tested concentration was already found in wastewaters effluents (Babić et al., 2010).

The wastewater was maintained in the systems for one week (hydraulic retention times normally used in full scale CWs), being replaced every week by new doped wastewater. Water level was maintained just above the substrate surface (flooding rate ≈100%). Every day the wastewater was recycled to prevent development of anoxic areas within roots' bed substrates.

Microcosms were kept under greenhouse conditions, subjected to environmental temperature variations (minimum 16 ± 2ºC and maximum 28 ± 8ºC) and environmental light exposure, along twelve weeks (April to July).

More details can be found in Carvalho et al. (2013).

2.2.2.Samples collection

Water and sediment samples were collected in planted microcosms at week 1 (W1), 2 (W2), 4 (W4), 8 (W8) and 12 (W12) and only at week 1, 2 and 4 in unplanted microcosms. The unplanted systems clogged at week 6.

Collected water samples were stored at -20 ºC for veterinary drugs evaluation as described in Carvalho et al. (2013) as well as for toxicity screening tests.

Collected sediment samples were stored at -20 ºC for further microbial and drugs analysis.

2.2.3.Antibiotic analysis

Antibiotics, TET and ENR, in wastewater samples were analyzed by high-performance liquid chromatography (HPLC), after a pre-treatment by solid – phase extraction (SPE) (Cavenati et al., 2012). The antibiotics were also analyzed in the roots' substrate bed using a previously optimized methodology: ultrasonic extraction with an appropriate solvent and analysis by HPLC (Carvalho et al., 2013b). More details can be found in Carvalho et al. (2013a).

2.2.4.Toxicity test

To evaluate wastewater toxicity ToxScreen test was performed. This test is based on the highly sensitivity variant of the luminescent bacterium *P. leiognathi* (test control). Thus, toxicity was evaluated through bacterial luminescence of the sample relatively to the test control (Ulitzur et al., 2002). For this evaluation, wastewater samples were previously centrifuged (15 min at 2500 rpm).

2.2.5.Microbial abundance

To estimate microbial abundance in sediments, Total Cell Counts (TCC) was obtained by the 4',6'-diamidino-2-phenylindole (DAPI) direct count method (Porter & Feig, 1980; Kepner & Pratt, 1994). For that, 2.5 mL of formaldehyde (4% (v/v)) were added to 0.25 g of homogenized sediment. Then, 2 drops of Tween (0.2 mm-filtered, 12.5% (v/v)) were added and samples were stirred for 15 min, resting for 15 min. These samples were then sonicated for 10 min, stirred again for 1 min and maintained overnight at 4 ºC. After this, 200 µL of the solution was added to 2.5 mL of saline solution (0.2 mm-filtered, 9 g L^{-1} NaCl) and 2 drops of Tween were added. Samples were then stained with DAPI and incubated in the dark for 15 min (Porter & Feig, 1980). Solutions were filtered

onto black Nucleopore polycarbonatefilters (0.2 mm pore size, 25 mm diameter, Whatman, UK) under gentle vacuum and washed with 5 mL of autoclaved 0.2 mm-filtered distilled water (fig.15 – A). Membranes were set up in glass slides (fig. 15 – B) and cells counted in an epifluorescence microscope (Leica DM6000B).

Fig. 15 - A: Vacuum filtration system; B: Sets of glass slides.

2.2.6. DNA extraction

DNA was extracted from 0.6 g wet weight of homogenized sediment samples using a CTAB (bromide-polyvinylpyrrolidone-b mercaptoethanol) modified extraction protocol described by Barrett et al. (2006). For a tube, 0.5 g of zirconia / silica beads 0.1 mm, 0.5 g beads 2.5 mm and 0.6 g of homogenized sediment were weighed. Therefore, 300 µl of NaH_2PO_4 (100 mM) and 300 µl of SDS solution were added and the samples were stirred for 20 minutes at maximum speed. Then, the samples were centrifuged for 3 minutes at 4ºC at 13.200 rpm and the supernatant was transferred to a new tube. Taking into account the number of samples, CTAB solution was prepared following the respective proportions: 1 mL CTAB Buffer requires 100 µl of PVP 10 % and 4 µl of BME. Of that solution, 200 µl of CTAB solution was added to each sample and they were left to incubate for 30 minutes at 60ºC at 100 rpm. After that, 1000 µl of chloroform isoamyl alcohol (24:1, v/v) were added and stirred for 15 seconds. The samples were centrifuged for 5 minutes at 4ºC at 13.200 rpm and the supernatant was transferred to a new tube. Then, 500 µl of chloroform isoamyl alcohol (24:1, v/v) were added, the samples stirred for 10 seconds and they were shaken in a horizontal stirrer at room temperature for 20 minutes. Thus, the samples were centrifuged for 5 minutes at 4ºC at 13.200 rpm and the supernatant was transferred for a new tube. Taking into account the volume of the supernatant, ammonium acetate, 7 M, was added in the following proportions: 560 µl of supernatant requires 310 µl of ammonium acetate, 7 M, in order to obtain a final concentration of 2.5 M. Therefore, the samples were centrifuged for 5 minutes at 4ºC at 13.200 rpm and the supernatant was transferred to a new tube. Then, 0.54 volumes of

isopropyl alcohol were added taking in to account the volume of supernatant (ex: 870 µl of supernatant requires 470 µl of isopropyl alcohol) and the samples were left to incubate overnight at -20 ºC. Therefore, the samples were centrifuged for 20 minutes at 4ºC at 13.200 rpm and the supernatant was rejected. The pellet was washed with 1000 µl of ethanol (70 %) and the samples were centrifuged for 20 minutes at 4ºC at 13.200 rpm. The supernatant was rejected, the samples centrifuged 5 minutes with open tubes and the pellet was resuspended in 25 µl of warm water. The quality of the extracted DNA was visualized in a 1.5% electrophoresis agarose gel.

2.2.7.Microbial community structure

Microbial community structure was evaluated by ARISA (Automated rRNA Intergenic Spacer Analysis), a technique that allows amplification of the 16S-23S intergenic spacer region in the rRNA operon (Fisher & Triplett, 1999). DNA was amplified using ITSF (5′ GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers set (Cardinale et al., 2004), which amplifies the ITS1 region in the rRNA. PCRs (polymerase chain reaction) were performed in duplicate 25 µL volumes containing between 0.5 µL and 1 µL of DNA, 0.4 µM of ITSF, 0.4 µM of ITSReub, Dream Taq PCR, Master mix 2x (Thermo Scientific), 2 mg/ml of bovine serum albumin (BSA). PCR program started at 95 °C for 2 min, followed by 8 cycles at 95 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min, then 30 cycles at 95° C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The last step ends at a temperature of 12 °C. PCR products were visualized in a 1.5 % electrophoresis agarose gel.

2.2.8.Electrophoresis agarose gel

To prepare the agarose gel, 1.5 g of agarose were mixed with 100 mL of TAE (1x) (1.5 % agarose gel) and the mixture was heated in the microwave for 4 minutes (2 minutes + 2 minutes). This time is necessary to fully dissolve the agarose. Then, 0.5 µl of SYBR® Safe was added and the gel was left to polymerize for 30 minutes. After that, the gel was placed in a horizontal electrophoresis cell (BIO RAD) and 5µl of each sample were loaded. The samples was turned on at 90 V for 30 minutes for extracted DNA and 90 V for 45 minutes for PCR products.

2.2.9.PCR products purification and quantification

PCR products were purified by UltraClean ® 15 Purification Kit (MO BIO Laboratories, Inc).

2.2.10. PCR products quantification

PCR products were quantified by Quant-it HsDNA assay kit and the Qubit fluorometer (Invitrogen). The work solution was made taking into account the following quantities: per each sample, 199 µL of Buffer and 1 µl of Qubit™ dsDNA HS reagent. The work solution was homogenized and distributed through the tubes. For the calibration, two standards were made: S1 and S2. For that, 190 µl the work solution and 10 µl of each standard were added in the respective tubes and waited up for 2 minutes. For the samples, 198 µl of work solution and 2 µl were added and waited up for 2 minutes. First, the equipment calibration was made and then, the analyses of the samples were performed. Sample fragments were run on a ABI3730 XL genetic analyzer at STABVIDA Sequencing Facilities (Lisbon, Portugal).

2.2.11. Statistical analysis

Sediment of each set of microcosms was analyzed in triplicate for all parameters being samples of each microcosm treated independently and the mean values and respective standard deviations calculated.

ARISA fragment lengths were evaluated by Peak Scanner™ version 1.0 Software (Applied Biosystems). Data was transferred to an excel sheet and transformed in a matrix of aligned fragments for further analysis in PRIMER 6 software package (version 6.1.11) (Clarke & Gorley 2006). In data analysis, fragments with Fluorescence Units below 50 were considered machine ‘‘background noise’’ and were not accounted for. Fragments of less than 200 bp were removed because they were considered to be too short ITS for bacteria. In Primer 6 software, to evaluate microbial community structure, the matrix was normalized using the presence/absence pre-treatment function and samples were analyzed using Bray–Curtis similarity method and then examined using a hierarchical cluster analysis. A samples clustering was generated using group average method and SIMPROF test was performed to test differences between generated clusters. A multidimensional scaling (MDS) plot was created using default parameters with a minimum stress of 0.01 to generate a configuration plot based on percent similarity.

To evaluate microbial community similarity, an analysis of similarities (two-way crossed ANOSIM, based on Bray-Curtis similarity) was performed using PRIMER 6

software (Clarke & Gorley, 2006). The ANOSIM is a permutation-based hypothesis statistical test, equivalent to univariate ANOVA, which tests for differences between groups of (multivariate) samples from different factors or experimental treatments (Danovaro et al., 2006).

Bacterial richness (or total number of species) and diversity indexes were obtained from ARISA profiles to have the ecological description of the bacterial community among samples. For this evaluation, peaks number was considered to represent species number and peak height was considered to represent the relative abundance of each bacterial species.

All statistical tests were performed using commercial software STATISTICA, version 12, StatSoft, Inc. (2013). For antibiotic analysis, TCC, toxicity, bacterial richness and diversity significant differences among samples were evaluated through a parametric one-way analysis of variance (ANOVA). Significant ($p < 0.05$) differences were detected by a multiple Tukey comparison test.

2.3. Results

2.3.1. Drugs removal efficiency

Significant reductions in drugs (ENR and TET) concentrations in wastewater along all one– week cycles were obtained in all systems tested (Carvalho et al., 2013). As observed in table 2 at least 94 % of TET and 98 % of ENR were removed from solution relatively to initial doping concentration (100 µg L^{-1}). No significant differences ($p > 0.05$) in removal efficiency were observed along time or between planted and unplanted systems.

Table 2 - Removal percentages (%) of enrofloxacin (ENR) and tetracycline (TET) from doped wastewater throughout time (mean and standard deviation, n=3). W1, W2, W4, W8, W12 – Weeks of experiment. Microcosms planted (P) or unplanted (X) with TET or ENR doped wastewater. Adapted from Carvalho et al., (2013).

	ENR		TET	
	P	X	P	X
W1	98.7 ± 0.3	99.3 ± 0.1	99.3 ± 0.4	99.1 ± 0.4
W2	98.5 ± 0.5	99.5 ± 0.3	98 ± 1	96.9 ± 0.2
W4	98 ± 1	99.5 ± 0.2	94 ± 5	98.4 ± 0.7
W8	99.0 ± 0.7	n.d	99.1 ± 0.8	n.d
W12	99.5 ± 0.2	n.d	98.9 ± 0.5	n.d

n.d. – not determined, system clogged after week 4.

Regarding drugs concentrations in roots' bed substrate none of tested antibiotic were detected, either in planted or unplanted systems, along time.

2.3.2. Toxicity

Toxicity of wastewater introduced in microcosms was higher than 99.9%, independently of antibiotic addition. Water collected from microcosms always presented significantly ($p < 0.05$) lower toxicity than introduced wastewater, with values ranging between 29 % and 95 % (table 3). Significantly higher toxicity percentages were obtained at week 4 for all treatments, which could be related with the presence of a non-identified toxic compound in the introduced wastewater that was not efficiently removed by the systems.

Generally, drugs presence in the wastewater did not interfere with systems capacity to remove toxicity. In some cases, the ability to remove toxicity was even

improved by veterinary drugs presence, a feature observed for unplanted systems in the first two weeks.

Also, in the first two weeks, a significantly lower ($p < 0.05$) toxicity for unplanted systems was observed comparing with planted ones. However, after four weeks, these differences were not evident.

Table 3 - Toxicity (%, mean and standard deviation, n=3) based on bacterial luminescence (ToxScreen test) in wastewater along the experiment. W1, W2, W4, W8, W12 – Weeks of experiment. Microcosms planted (P) or unplanted (X) with not doped wastewater (Control) and with tetracycline (TET) or enrofloxacin (ENR) doped wastewater. Adapted from Carvalho et al., (2013).

	Control		TET		ENR	
	P	X	P	X	P	X
W1	85 ± 1 [b]	73 ± 3 [b,]	87 ± 1 [b,]	52 ± 4 [a, b]	58 ±7 [a,b]	29 ± 6 [a, b]
W2	60 ± 2 [b, c]	47 ± 5 [b, c]	47 ± 16 [c]	31 ± 8 [a, c]	39 ± 12 [a]	31 ± 5 [a]
W4	96.3 ± 0.3 [b, c]	90.4 ± 0.2 [b, c]	94 ± 2 [c]	95 ± 2 [a, c]	94 ± 4 [c]	n.d.
W8	81 ± 1 [c]	n.d	75 ± 6 [c]	n.d	75 ± 3 [a,c]	n.d
W12	86 ± 2 [c]	n.d	78 ± 5	n.d	81 ± 2 [a]	n.d

n.d – not determined, systems clogged after week 4.

a - significant differences comparing with respective control ($p < 0.05$);

b - significant differences comparing planted and unplanted systems ($p < 0.05$);

c - significant differences along time ($p < 0.05$), comparing one week with the previous one.

2.3.3.Microbial abundance

Microbial abundance, TCC estimated in sediments collected weekly from each microcosm, ranged from 106 to 107 log10 g^{-1}wet sediment (fig. 16).

Comparing each treatment with the respective control, no significant differences in TCC were observed ($p > 0.05$) with a single exception. The same was observed when comparing throughout time each treatment, although there was a tendency for TCC to increase in unplanted systems along time. Regarding planted and unplanted systems, results showed no significant differences, but there was a tendency for higher TCC values in planted systems.

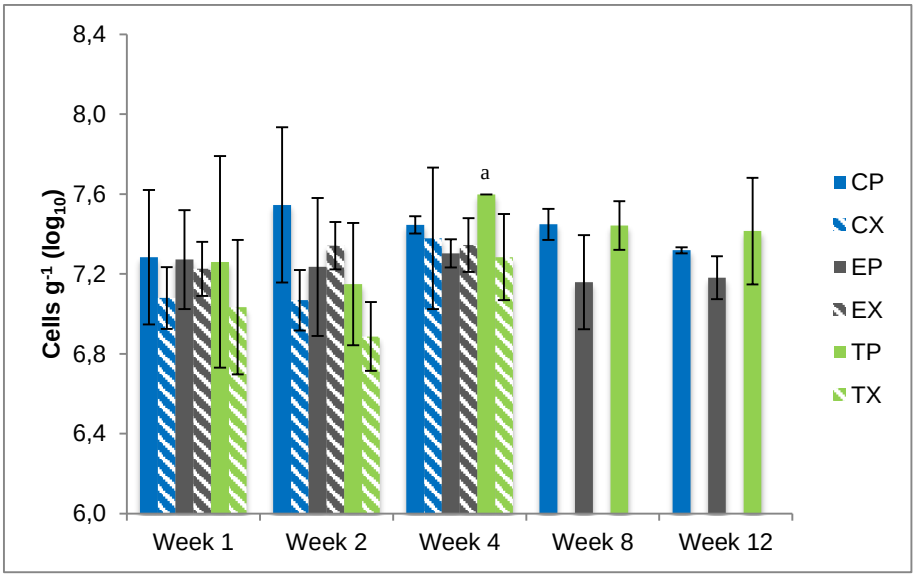


Fig. 16 - Microbial abundance (mean and standard deviation, n=3) in sediments along the experiment. CP – Planted Control; CX – Unplanted Control; TP – Planted TET treatment; EP – Planted ENR treatment; TX - Unplanted TET treatment; EX – Unplanted ENR treatment. a - significant differences comparing with respective control ($p < 0.05$).

2.3.4. Bacterial richness and diversity

For each sediment sample, bacterial richness and diversity indexes were calculated from ARISA profiles. Results showed no significant differences ($p > 0.05$) comparing each treatment with the respective control for bacteria richness (fig. 17) and diversity indexes (fig. 18) with few exceptions. Comparing planted and unplanted treatments, generally no significant differences ($p > 0.05$) were observed in terms of bacterial richness or diversity, although, in week 2 and 4, there was a higher richness and diversity ($p < 0.05$) in unplanted systems when wastewater was doped with ENR.

In addition, no significant differences ($p > 0.05$) were observed for each treatment through the time, in terms of bacterial richness or diversity (with a single exception).

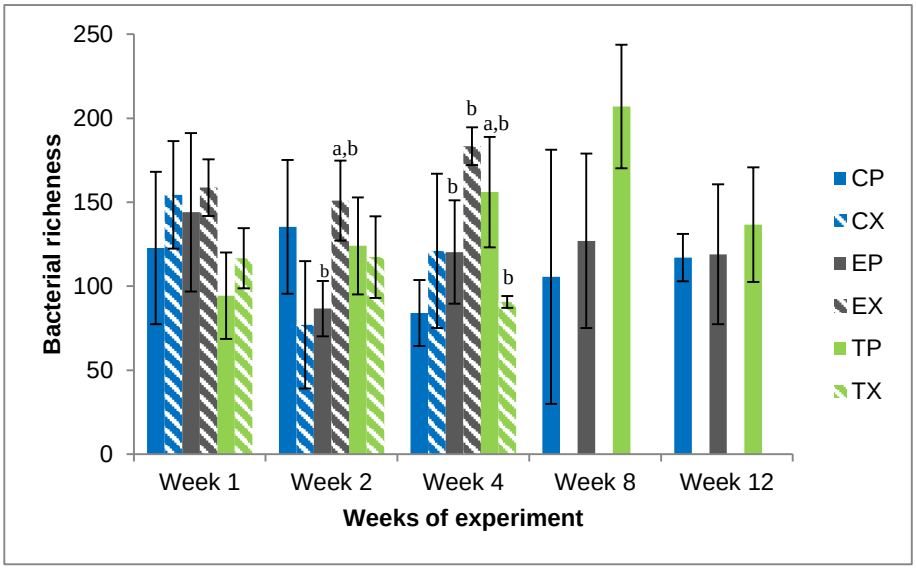


Fig. 17 - Bacterial richness in sediments along the experiment. CP – Planted Control; CX – Unplanted Control; TP – Planted TET treatment; EP – Planted ENR treatment; TX - Unplanted TET treatment; EX – Unplanted ENR treatment. a - significant differences comparing with respective control ($p < 0.05$); b - significant differences comparing planted and unplanted systems ($p < 0.05$).

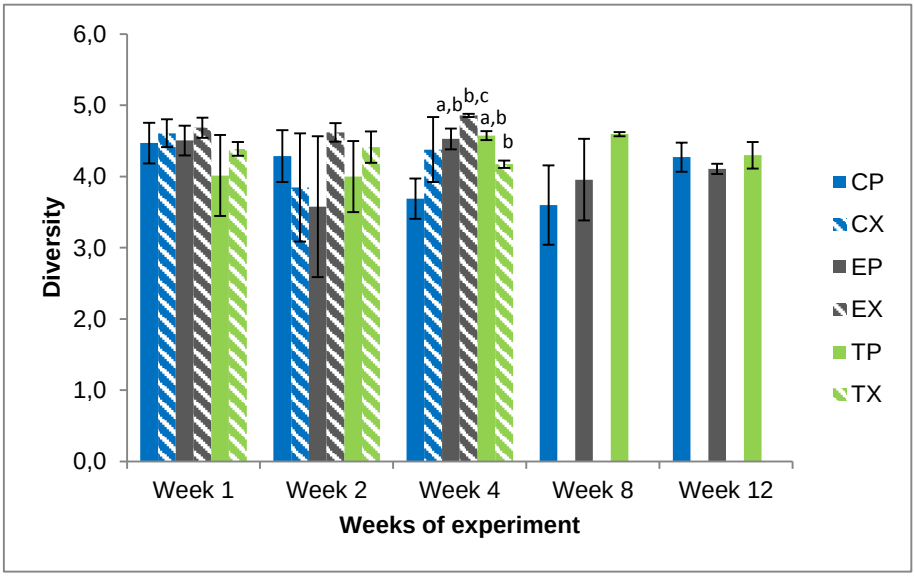


Fig. 18 - Bacterial diversity in sediments along the experiment. CP – Planted Control; CX – Unplanted Control; TP – Planted TET treatment; EP – Planted ENR treatment; TX - Unplanted TET treatment; EX – Unplanted ENR treatment. a - significant differences comparing with respective control ($p < 0.05$); b - significant differences comparing planted and unplanted systems ($p < 0.05$); c - significant differences along time ($p < 0.05$), comparing one week with the previous one.

2.3.5. Bacterial community structure

ARISA analysis was performed in initially collected sediment and in three replicates from each treatment collected from different microcosms. For each sample, ARISA fragments lengths (ALF) profiles were obtained. These fragments corresponded to total number of peaks and thus to different bacteria phylotypes. However, the difference in their genetic structure which is the distribution of the different phylotypes among the different samples is the really important feature.

To understand bacterial community evolution along the experiment, MDS analysis was performed based on similarity between samples obtained from ARISA analysis. To simplify results interpretation, 3 MDS were created, trying to visualize sources of variation between samples: variation in planted microcosms (control, enrofloxacin and tetracycline) along the 12 weeks of experiment (fig. 19 - A); variation between TET and control treatments in planted and unplanted microcosms (fig. 19 - B); and variation between ENR and control treatments in planted and unplanted microcosms (fig. 19 - C). Additionally, analysis of similarity (two-way crossed ANOSIM) was performed to identify significant differences between groups of samples (table 3).

Regarding planted systems exposed to different treatments (control, ENR and TET) along the 12 weeks of experiment (fig. 19 - A), analysis of similarity (table 4 - A) showed a significant effect of both time and treatment, being time of exposure the most important factor defining bacterial community structure, followed by the type of treatment. Concerning TET and control treatments in planted and unplanted systems along the 4 weeks of experiment (fig. 19 - B), analysis of similarity (table 4 - B) showed once again time of exposure was the most important factor defining bacterial community structure, followed by the type of treatment, whereas plants' presence represented only a small contribution to this variation. On the other hand, regarding ENR and control treatments in planted and unplanted systems along the 4 weeks of experiment (fig. 19 - C), analysis of similarity (table 4 - C) showed plants' presence was an important factor in bacterial community structure definition, immediately after the treatment type, whereas time of exposure had only a small contribution to this variation.

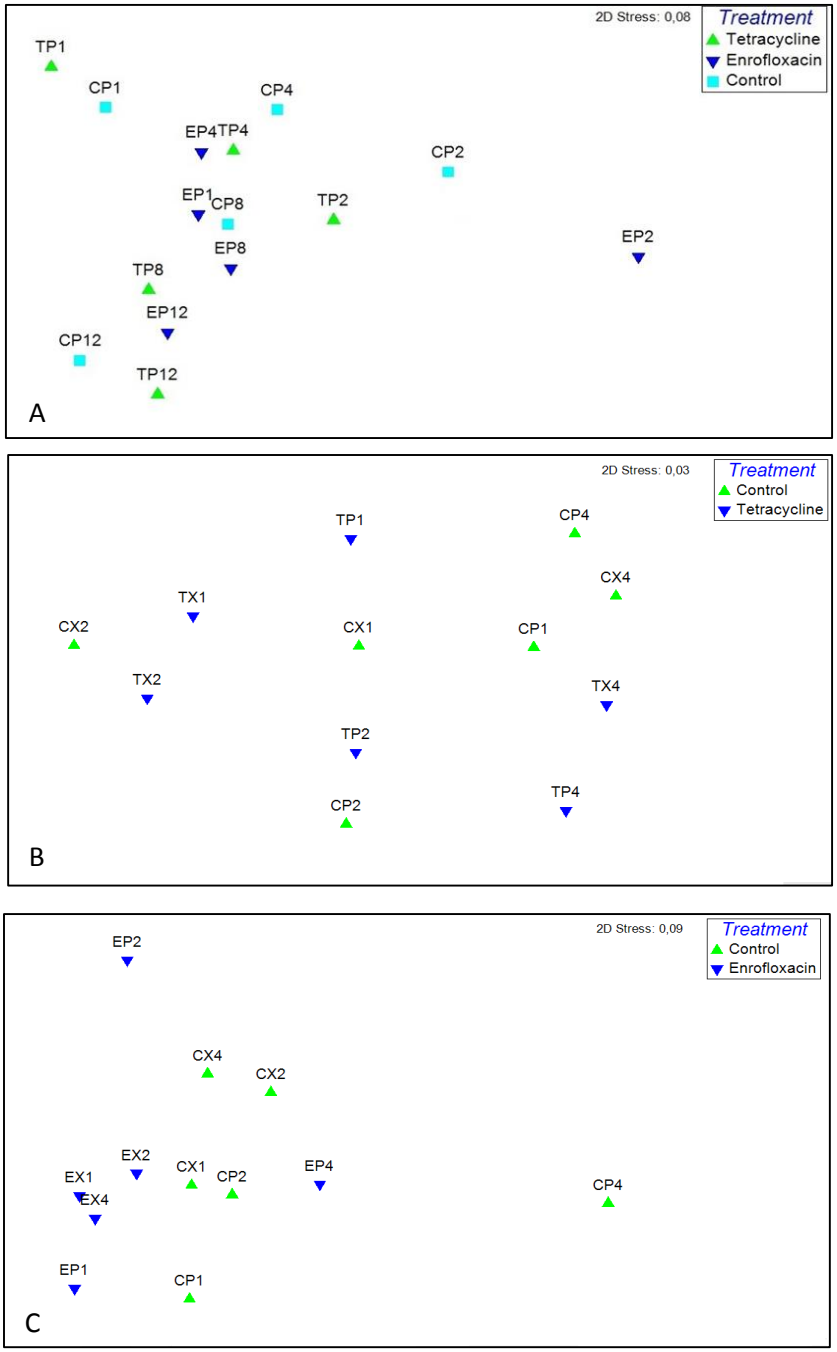


Fig. 19 - Multidimensional scaling (MDS) ordination based on Bray–Curtis similarities on the presence/absence matrix obtained from ARISA fingerprints of bacterial communities along the experiment. A - Planted systems along time of experiment; B – Tetracycline vs control treatment in planted and unplanted systems; C – Enrofloxacin vs control treatment in planted and unplanted systems. CP – Planted Control; CX – Unplanted Control; TP – Planted TET treatment; EP – Planted ENR treatment; TX - Unplanted TET treatment; EX – Unplanted ENR treatment. The three replicates were averaged before analysis.

Table 4 - Global test two-way crossed ANOSIM test for different treatments, time of exposure and plant effect, based on ARISA results from: A - Planted systems along time of experiment; B – Tetracycline vs control treatment in planted and unplanted systems; C – Enrofloxacin vs control treatment in planted and unplanted systems.

A - Planted systems along time of experiment		
Treatment type vs Time of experiment	**Statistic value (R)**	**Significance level**
Treatment	0.550	0.1
Time	0.890	0.1
B – Tetracycline vs control treatment in planted and unplanted systems		
Treatment type vs Time of experiment	**Statistic value (R)**	**Significance level**
Treatment	0.511	0.1
Time	0.640	0.1
Time of experiment vs Plant presence		
Time	0.576	0.1
Plant	0.323	0.1
Treatment type vs Plant presence		
Treatment	0.520	0.1
Plant	0.237	2.5
C – Enrofloxacin vs control treatment in planted and unplanted systems		
Treatment type vs Time of experiment	**Statistic value (R)**	**Significance level**
Treatment	0.364	0.1
Time	0.255	0.2
Time of experiment vs Plant presence		
Time	0.225	1.2
Plant	0.487	0.1
Treatment type vs Plant presence		
Treatment	0.655	0.1
Plant	0.580	0.1

2.4. Discussion

Constructed wetlands are being considered a potential technology to remove pharmaceuticals from wastewater effluents, but their ability to improve water quality depends greatly on their microbial communities. In the present work, the response of the microbial community from CWs microcosms tested for the removal of two veterinary antibiotics (ENR and TET) from livestock industry wastewater was investigated. Not only antibiotics effects, but also plants effects on microbial communities were evaluated by using systems unplanted and planted with P. australis.

Constructed wetlands removal efficiency was relatively stable along time, with removals from doped wastewater higher than 98 % for ENR and 94 % for TET. No significant differences were observed between unplanted and planted systems, but unplanted systems clogged after 4 weeks of experiment, pointing to the importance of plants for systems stability.

In addition, CWs were able to decrease wastewater toxicity, independently of the antibiotics presence. In fact, wastewater toxicity decreased from 99.9% before treatment to values between 29 % and 95 % after treatment. So, CWs efficacy to remove other toxic compounds, improving the water quality beyond the removal of pharmaceuticals, was confirmed.

Constructed wetlands microbial community response to the different treatments was evaluated in terms of total abundance, bacterial diversity indexes and bacterial community structure.

In general, no significant differences were observed in terms of bacterial abundance, richness or diversity among different treatments (without drugs addition or with TET or with ENR) or along the time of experiment. Berglund et al. (2014) also observed no effect on bacterial diversity after continuous exposure to a mixture of 12 antibiotics in experimental wetlands. However, there is an increasing body of evidence documenting a reduction of bacterial diversity in soils contaminated with antibiotics (Jechalke et al., 2014; Kong et al., 2006; Ollivier et al., 2013). Also, in a batch reactor experiment, Zhang et al. (2013) observed a decreased in the microbial diversity indexes at 100 µg L^{-1} of TET, the same concentration used the in present study. A high level of diversity is considered an important feature of ecosystem integrity as it implies functional redundancy, acting as a genetic and functional reservoir that increases community resilience to disturbance (Bissett et al., 2007). Therefore, loss of community diversity has been used to indicate a decline in ecosystem function (Allison & Martiny, 2008), a feature not observed in the present study. In fact, results indicated that CWs, along with maintaining their bacterial abundance, richness and diversity, maintained drugs and

toxicity removal efficiency.

Diversity indexes are species independent methods of community analysis that, although less sensitive in detecting changes than multivariate methods, some value judgment can be attached to the changes observed (Warwick & Clarke, 1991). Nevertheless, communities with completely different composition can present the same values for these indexes. On the other hand, multivariate methods have advantages of great sensitivity and specificity of response, despite being more difficult to interpret in terms of value judgments (detrimental or otherwise). Therefore, bacterial community structure was assessed. Shifts on bacterial community composition were analyzed by ARISA, a DNA fingerprinting technique that allows the rapid assessment of the genetic structure of complex communities in diverse environments (Ranjard et al., 2001; Hewson & Fuhrman, 2004; Danovaro et al., 2009), and of the extent of changes caused by environmental disturbances (Malik et al., 2008, and references therein).

The multivariate analysis of all generated ARISA profiles allowed detection of several differences in terms of community structure between treatments. Analysis of similarity showed time of exposure was the most important factor in defining bacterial community structure, followed by the type of treatment, whereas plant presence explained part of the differences observed between ENR and control treatments in the first 4 weeks of experiment.

The fact that time of exposure was the most important structuring factor for bacterial community indicates community was in an adapting process, independently of antibiotics presence. This must be related with the fact sediments used in this experiment were collected from a natural environment and were exposed to wastewater collected in a pig farm with a very high organic load. Therefore, the original bacterial community had to adapt to very different environmental conditions, appearing to be in an adaptation process along the 12 weeks of experiment.

Second most important factor for bacterial community structure definition was the type of treatment, i.e. the presence or absence of one of the tested antibiotics (control, TET or ENR). One main mechanism for drugs removal in CWs systems is adsorption to microcosms supporting matrix (Dordio et al., 2010) (in the microcosms assembled this matrix had three different layers), which leads to microbial communities' exposure to drugs. Although none of the drugs was detected in roots' bed substrate, and drug adsorption in this layer could not be confirmed, doped wastewater was embedded always in the sediment (flooding rate ≈100%). Therefore, microbial communities were exposed to TET or ENR. Several authors reported veterinary antibiotics effects on structure and functioning of soil microbial communities (Jechalke at al., 2014 and references therein). Hammesfahr et al. (2008) reported changes in microbial community structure after

application of manure containing sulfadiazine in soils and observed delayed and prolonged effects on microbial community structure which increased over time. Reichel et al. (2013) also reported effects of slurry from sulfadiazine and difloxacin medicated pigs on soil microbial communities. In addition, in a batch reactor experiment considerably changes in microbial community structure were observed in the presence of 100 μg L^{-1} of TET (Zhang et al., 2013), the same concentration used in the present study. Regarding possible ENR effects on soil or sediment microbial community no data in the literature was, however, found. So, present results indicated the two tested antibiotics can affect bacterial communities' structure although not affecting bacterial richness or diversity.

The third factor responsible for microbial community differentiation was plants' presence, although this effect could only be detected when comparing ENR and control treatments. Plants can exert an important influence and can shape microbial communities structure and composition (Ribeiro et al., 2013).In the present study, for ENR treatment there was also a slight difference in bacterial richness and diversity between planted and unplanted systems. Plant influence can be carried out, for instance, by enhancing their activity through root exudation (Bais et al., 2006, Koranda et al., 2011). Root exudates composition and quantity depend on several factors, including plant species (Bais et al., 2006). In addition, plants themselves can be affected by drugs presence and respond differently to different drugs. For instance, previous results for P. australis (Carvalho et al., 2012) pointed to some plant stress due to ENR exposure. In fact, ENR may generate both toxic effect and hormesis to plants, which are related to plant drug uptake (Fatta-Kassinos et al., 2011). Although in current CWs microcosms no plant induced stress and phytotoxicity signs were observed in the long run, control systems, without drugs addition, were the first to stabilize chlorophylls contents in plant leaves (Carvalho et al., 2013). Therefore, plants adaption to drugs presence could also influence bacterial community structure.

Changes in microbial community structure can affect ecological functions of soil ecosystems, like biomass production and N-transformation processes (Thiele-Bruhn & Beck 2005; Kotzerke et al., 2008). Nevertheless, other studies revealed community shift is not necessarily mirrored by an altered soil functioning but masked by functional redundancy sustained by a structurally changed microbial community (Hammesfahr et al., 2008). In the present study, despite changes in bacterial community structure, CWs microcosms maintained its depuration capacity, reducing toxicity and significantly removing drugs from provided wastewater.

2.5. Conclusions

Microbial community dynamics associated with veterinary antibiotics removal from livestock industry wastewater was studied in CWs microcosms.

No significant differences were observed in terms of microbial abundance, bacterial richness or diversity either among different treatments (with or without TET or ENR) or along the experimental time. However, multivariate analysis of ARISA profiles showed several differences in terms of community structure among treatments. In fact, time of exposure was the most important factor in defining bacterial community structure, followed by the type of treatment, whereas plants presence explained part of the differences observed between ENR and control treatments.

Constructed wetlands microbial communities were able to adapt without significant changes in their diversity or depuration capacity. In fact, CWs drugs removal efficiency was relatively stable along time, with removals from doped wastewater higher than 98% for ENR and 94% for TET. In addition, CWs were able to reduce wastewater toxicity, independently of antibiotics presence.

This study highlights CWs importance for removal of veterinary antibiotics found in livestock wastewaters, showing promising results in its application in the remediation of the environmental impact of livestock industry. However, more studies are needed to understand the complex reactions/mechanisms occurring in antibiotics removal.

Chapter 3

Response of a salt marsh microbial community to antibiotic contamination

3. Response of a salt marsh microbial community to antibiotic contamination

3.1. Introduction

Estuaries are among the most productive ecosystems on Earth (Hewson, & Fuhrman, 2004), but also among of the most sensitive and, consequently, the most difficult to recover (Mucha et al., 2011).

Estuaries have a range of different wetland habitats within, including salt marshes (fig. 20). Thomas et al. (2014) defined salt marshes as “highly productive coastal ecosystems found in intertidal areas and vegetated by salt tolerant non-woody plants”.

Fig. 20 - Example of a salt marsh in Lima Estuary (North of Portugal) (http://tablet.avesdeportugal.info/images/Veiga_S._Sim_o_1.jpg).

Polluted estuaries have been reported of all over the world and their sediments can be considered both as sinks and sources of contaminants (Mucha et al., 2011). With the excessive evolution in coastal areas, estuaries now present a wide variety of chemical contaminants (Sun et al., 2012), like emerging pollutants (Stewart et al., 2014) persistent organic pollutants (POP) and metals (Pan & Wang, 2012). These compounds can enter in the water system through industrial discharges; urban and farmlands discharges from WWTP’s; storm drains; and atmospheric deposition (Sun et al., 2012). Depending on their physicochemical properties, contaminants, can accumulate in estuarine sediment; can be concentrating in the water or bioaccumulated by sediment-dwelling organisms (Meador et al., 1995; Sun et al., 2012).

Bioremediation, the use of natural biological processes for ecosystem recovery, can arise as a less damaging and more cost effective method when compared with

traditional techniques such as soil washing, incineration or disposal landfills (Mucha et al., 2011). Microorganism's activity can improve degradation of organic pollutants transforming them into less toxic and less bioavailable products (Ribeiro et al., 2011).

The interactions between microorganisms and salt marsh plants can be determinant in the contaminant degradation. Sediment and plant rhizosphere present in estuarine ecosystems are very rich in microorganisms that can be stimulated by plant root exudates (Bais et al., 2006; Prosser et al., 2006; Salvato et al., 2012). On the other hand, the plant can play an important role in the bioremediation of organic pollutants by enhancing microbial degradation through specific microenvironments for pollutant-degrading microorganism (Johnson et al., 2004).However, low bioavailability of the pollutants due to adsorption to soil particles can be a potential obstacle to an effective biodegradation(Johnson et al., 2004). In addition, interactions between plant and microorganisms are very complex (Prosser et al., 2006) and can be influenced by bulk rhizosphere carbon flow, by modifications generated by signaling molecules and blockers of signals (Prosser et al., 2006) and by plant species (Ribeiro et al., 2011).

Biodegradation pathways of organic compounds in water systems depend on temperature, availability of organic and inorganic nutrients, type of sediment and presence of oxygen. In addition, biodegradation rates are controlled by organic compound concentration (Ingerslev et al., 2001).

Physico-chemical reactions that occur in wetlands can also improve remediation of contaminants. (Williams, 2002). The high productivity of wetlands and high rate of photosynthesis and transpiration can enhance phytoremediation actions (Williams, 2002).

The aim of this study was to evaluate, in the laboratory, the response of a salt marsh plant-microorganisms association to a contamination with a veterinary antibiotic. For that a salt marsh plant (*P. australis*) and respective rhizosediment were collected in a temperate estuary (Lima estuary, NW Portugal) and exposed for 7 days to ENR under different nutritional conditions. Response was evaluated in terms of ERN removal and changes in terms of microbial community structure and abundance.

3.2. Material and methods

3.2.1.Sampling

Plant (*P. australis)* and the respective rhizosediment (sediment around plant roots) were collected in Lima River Estuary (North of Portugal) in November 2013. The sediment was separated from the roots and kept aside for preparation of the experiments. One fraction of the sediment was maintained at -20ºC for posterior microbial community analysis.

Estuarine water was collected upstream the site where plants were collected to avoid larger amount of salinity.

3.2.2.Laboratory experiments

At the beginning of the experiment, elutriate was prepared according to Environmental Protection Agency protocols (USEPA, 1991), 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. In total, 32 flasks were prepared. To prepare the flasks without sediment (elutriate flasks), solutions from 8 of the 32 flasks were centrifuged and filtrated sequentially through 0.8 µm and 0.45 µm pore size filters (cellulose nitrate membrane, Millipore), to remove particulate suspended matter (except colloids) and to reduce the presence of microorganisms.

The systems were set up in glass flasks, like is represented in fig. 21. The flasks were divided in 4 treatments, all containing sediment: (1) the control (C), only with elutriate and sediment; (2) ENR (100 µg L^{-1}) treatment (E) (3) ENR + nutrients (1008 µg L^{-1} KH_2PO_4; 3790 µg L^{-1} KNO_3) treatment (EN) (4) ENR + Nutrients + $C_6H_{12}O_6$ (180 µg L^{-1}) treatment (ENC). For each treatment, planted (with *P. australis*) (SP) and unplanted (S) systems were prepared in flasks with sediment and elutriate. In the planted ones, plant roots were completely submerged. For ENR treatment, an additional set of flasks was prepared with elutriate without sediment, both with (E A) and without plants (E AP).

Each flask was wrapped in aluminum foil to avoid light degradation of ENR due to light penetration into the substrate. The flasks were exposed to natural day: night regime with natural sunlight for 1 week. In the middle of the week, a second doping of 100 µg L^{-1} of ENR was performed.

At the end of the experiment, all elutriate samples were collected from each flask and stored at - 20 ºC for further quantification of ENR. Sediment samples were collected from each flask, also stored at -20ºC, for further analysis in terms of microbial community structure and evaluation of levels of ENR.

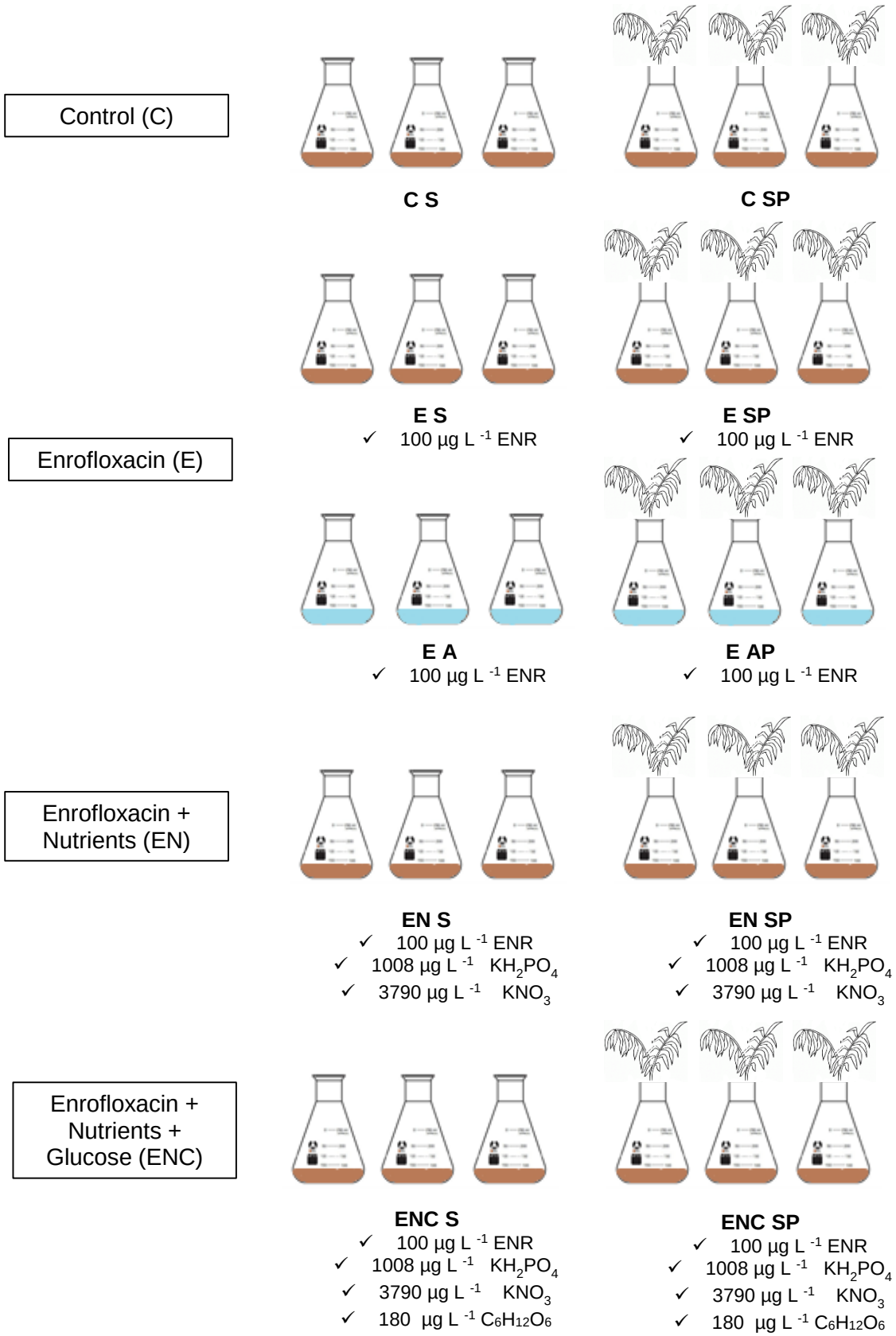


Fig. 21 - Scheme of the experiment for each treatment (C – control; E – Enrofloxacin; EN – Enrofloxacin + nutrients; ENC – Enrofloxacin + nutrients + glucose), either in the absence (A or S) or in the presence (AP or SP) of plants. A – filtered elutriate ; S –elutriate with sediment.

3.2.3.Samples preparation

SPE (solid-phase extraction) was performed to concentrate the ENR present in solutions collected from the experiment and to clean the matrix as described in Carvalho et al. (2013b). (fig. 22). At the beginning, samples were filtrated through 0.45 µm pore size membrane filters and the pH was adjusted to 2. SPE cartridges, Oasis HLB (60 mg, 3 mL) cartridges from Waters Corporation (Millford, MA, USA), were conditioned with 5 mL of methanol followed by 5 mL of deionized water using a vacuum manifold system (Supelco, Spain) connected to a vacuum pump. Then, the samples were passed through the pre-conditioned cartridges. Afterwards, the loaded cartridges were washed with 5 mL of a methanol/water mixture (5:95 v/v) and dried out under vacuum conditions for 30 min. Then, the elution was performed with 5 mL of a methanol/formic acid mixture (96:4 v/v). After that, the extracts were evaporated to dryness under a nitrogen stream at 35 °C. The residue was dissolved in 1.0 mL of the HPLC mobile phase (water/formic acid, 99:1, v/v).

Fig. 22 - SPE procedure.

To evaluate the levels of ENR in the sediment, sediments extractions were performed (fig. 23). Sediment was lyophilized, being afterwards homogenized. Then, 2 g of sediment were weighed for an amber vial and 10 mL of methanol/acetone mixture (95:5; v/v) were added. Vials were placed in an ultrasonic bath for 15 minutes, then centrifuged for 5 minutes (2500 rpm) and all of the supernatant was collected for another vial. 10 mL of methanol/acetone mixture (95:5; v/v) were added again to the remaining sediment and the same procedure was applied. The two supernatants were combined and evaporation (in N_2 flux) of the collected extract (approximately 14 mL) was

performed. Then the residue was dissolved in 1.0 mL of mobile phase (water / formic acid, 99:1, v/v).

Fig. 23 - Several steps of sediment extraction.

3.2.4.Antibiotics analysis

Enrofloxacin was analyzed in a Beckman Coulter equipment (HPLC-system gold). 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 Luna column (Phenomenex, UK).

Two mobile phases (water /formic acid, 99:1, v/v) and acetonitrile (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), keeping isocratic conditions for 2 min, followed by a 10 min gradient to 50 % of eluent A (50% of eluent B (acetonitrile). Then, gradient to 100 % of eluent A were reached again in 10 min, with a re-equilibration time of 2 min to restore the column. Flow rate gradient started with 0.5 mL min^{-1}, which was maintained for 2 min and then was increase to 1 mL min^{-1}.

The sample injection volume was set at 50 µL and the detector signal was monitored at λ = 280 nm.

A calibration was performed with aqueous standard solutions. The standard solutions with 0.3, 0.5 and 0.7 mg L^{-1} were made in 10 mL graduated flasks with the

proportions presented in table 5. After that, 1 mL of each standard was transferred for 2 mL HPLC vials.

The remaining standard solutions (1, 3, and 5 mg L^{-1}) were directly prepared in 2 mL HPLC vials (table 6)

Table 5 - Standard solutions of 0.3, 0.5 and 0.7 mg L^{-1} of ENR.

Concentration (mg/L)	Mobile phase (H2O/ formic acid 99:1 v/v) mL	Standard solution (40 mg L^{-1})Volume mL
0.3	9.925	0.075
0.5	9.875	0.125
0.7	9.825	0.175

Table 6 - Standard solutions of 1,3 and 5 mg L^{-1} of ENR.

Concentration (mg/L)	Mobile phase (H2O/ formic acid 99:1 v/v) mL	Standard solution (40 mg L^{-1}) Volume mL
1	0.975	0.025
3	0.925	0.075
5	0.875	0.125

Recovery percentages in solution doped with a known amount of ENR before HPLC analysis were 87±14% for elutriated samples and 95±11 % for sediment samples.

Recoveries, evaluated by doping elutriate solutions with a known amount of ENR before filtering and SPE, were around 29±2 %.

The limits of detection (LOD), considering the SPE pre-concentration step in this work (50 mL of sample in SPE) were 3 µg L^{-1} of ENR for elutriates samples. For sediment sample, LOD were 0.075 µg g^{-1} of ENR.

3.2.5.Microbial abundance

The microbial abundance was determined as described in chapter 2 – Material and Methods – section 2.2.5.

3.2.6.DNA extraction

DNA was extracted from 0.5 g wet weight of homogenized sediment samples using Power Soil Extraction Kit (Mo Bio Laboratories, Inc). The quality of extracted DNA was evaluated in a 1.5% electrophoresis agarose gel (Chapter 2 - Material and Methods – section 2.2.8).

3.2.7.Microbial community structure

Microbial community structure was evaluated by ARISA (Automated rRNA Intergenic Spacer Analysis), a technique that allows the amplification of the 16S-23S intergenic spacer region in the rRNA operon (Fisher & Triplett, (1999). In the ARISA method, the DNA was amplified using ITSF (5′ GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers set (Cardinale et al., 2004), which amplifies the ITS1 region in the rRNA. PCRs (polymerase chain reaction) were performed in duplicate 25 µL volumes containing between 0.5 µL and 1 µL of DNA, 3x Taq PCR Buffer, 2 mM MgSO4, 0.4 µM of ITSF, 0.4 µM of ITSReub , 0,2 mM dNTPs, 1 mg ml^{-1} of bovine serum albumin (BSA) and 2.5 U Taq DNA polymerase. The PCR program started at 94°C for 2 min, followed by 30 cycles of 94° C for 45s, 55°C for 30 s, 72°C for 2 min and a final extension at 72°C for 7 min. The last step ends at a temperature of 12°C. The PCR products were visualized in a 1.5% electrophoresis agarose gel (Chapter 2 - Material and Methods – section 2.2.8).

3.2.8.PCR products quantification and purification

The purification and quantification were the same described in detail in Material and Methods – section methods chapter 2 - 2.2.9 and 2.2.10, respectively.

3.2.9.Statistical analysis

Statistical analysis was performed as described in chapter 2 - Material and Methods – section 2.2.11.

3.3. Results

3.3.1.Systems after one week of experiment

After one week of experiment, all systems were disassembled**.**

The planted flasks without sediment (E AP) had an odor which indicates the beginning of system decomposition however, the plant and roots were not deteriorated (fig. 24 – B).The unplanted flasks (E A) were apparently identical comparing with the initial ones (fig. 24 – A).

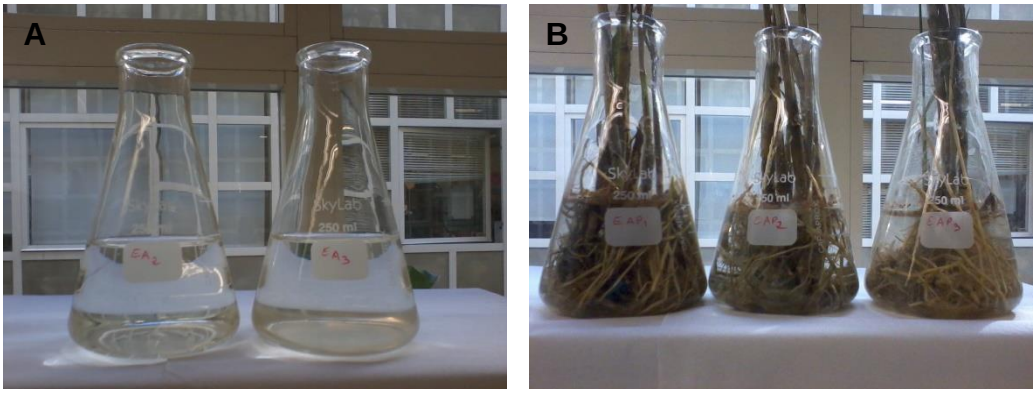


Fig. 24 - A: Unplanted flasks without sediment at the end of experiment (E A); B: Planted flasks without sediment at the end of experiment (E AP).

The unplanted controls (C S) presented brownish turbid water with suspended particles (fig. 25 – A). The planted controls (C SP) had an intensive odor which indicates the system decomposition. The plant and roots were deteriorated and the sediment was almost black (fig. 25 – B).

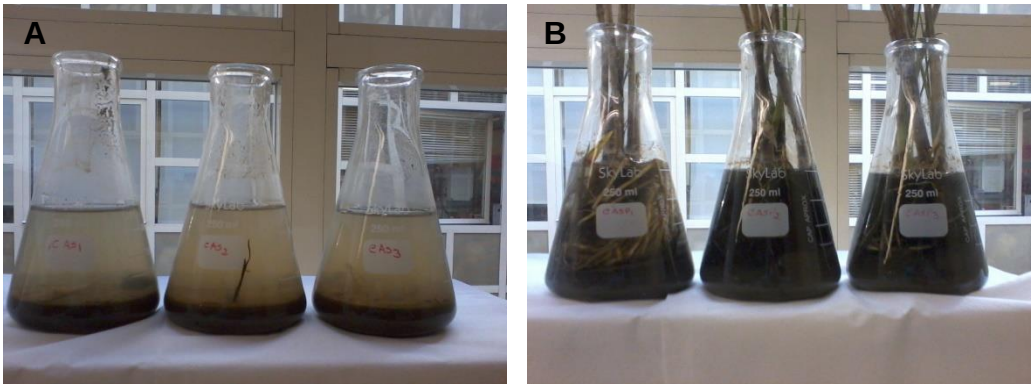


Fig. 25 - A: Unplanted controls at the end of experiment (C S); B - Planted controls at the end of experiment (C SP).

Regarding unplanted ENR systems (E S), they presented brownish turbid water with suspended particles (fig. 26 – A). The planted ENR systems (E SP) presented higher odor compared with planted controls. The plant and roots were deteriorated and the sediment was completely black (fig. 26 – B).

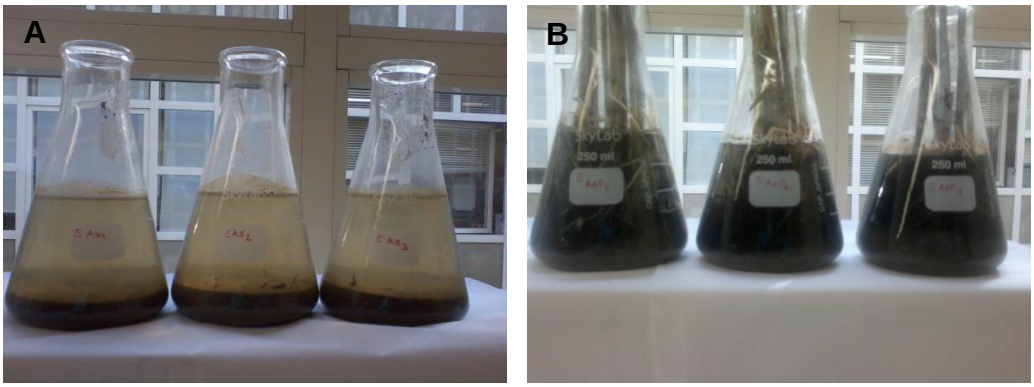


Fig. 26 - A: Unplanted ENR treatment at the end of experiment (E S); B - Planted ENR treatment at the end of experiment (E SP).

Unplanted ENR systems with nutrients (EN S) showed brownish water with suspended particles but slightly more transparent comparing with the previous treatments (fig. 27 – A). The planted ENR systems with nutrients (EN SP) presented dark brown sediment and the odor was not detected. The plant and roots were not deteriorated (fig. 27 – B).

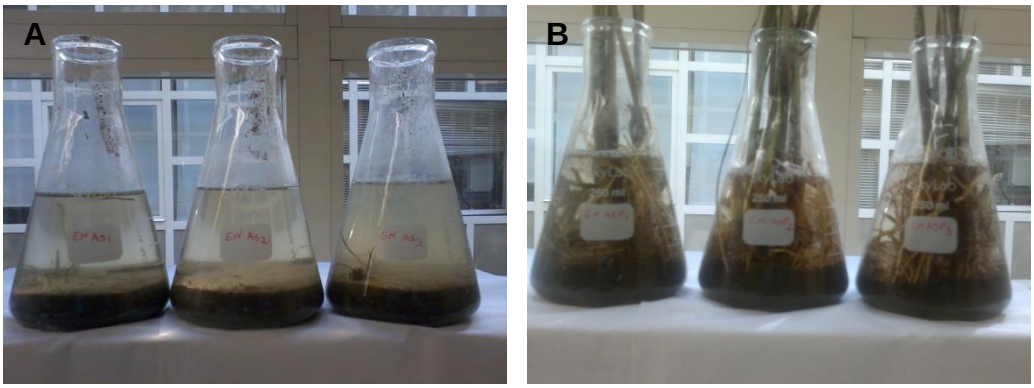


Fig. 27 - A: Unplanted ENR treatment with nutrients at the end of experiment (EN S); B - Planted ENR treatment with nutrients at the end of experiment (EN SP).

Regarding unplanted ENR systems with nutrients and glucose (ENC S), they presented brownish water with suspended particles and more transparent comparing with the unplanted ENR systems with nutrients (fig. 28 – A). The planted ENR systems with nutrients and glucose (ENC SP) presented brown sediment and no odor was detected. The plant and roots were not deteriorated (fig. 28 – B).

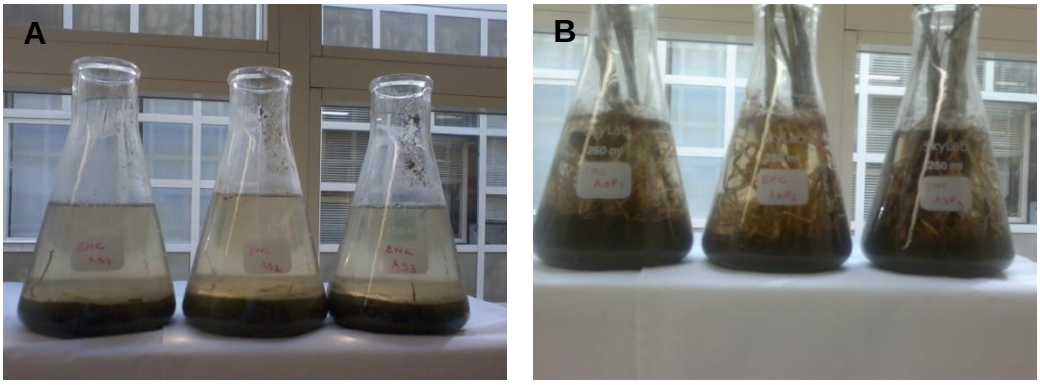


Fig. 28 - A: Unplanted ENR treatment with nutrients and glucose at the end of experiment (ENC S); B - Planted ENR treatment with nutrients and glucose at the end of experiment (ENC SP).

3.3.2.Levels of antibiotics

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

3.3.2.1. Elutriate solutions

Regarding treatments with elutriate only (fig. 29), significantly different ENR concentrations were observed for planted and unplanted treatment ($p < 0.05$), with higher concentrations in the unplanted treatment.

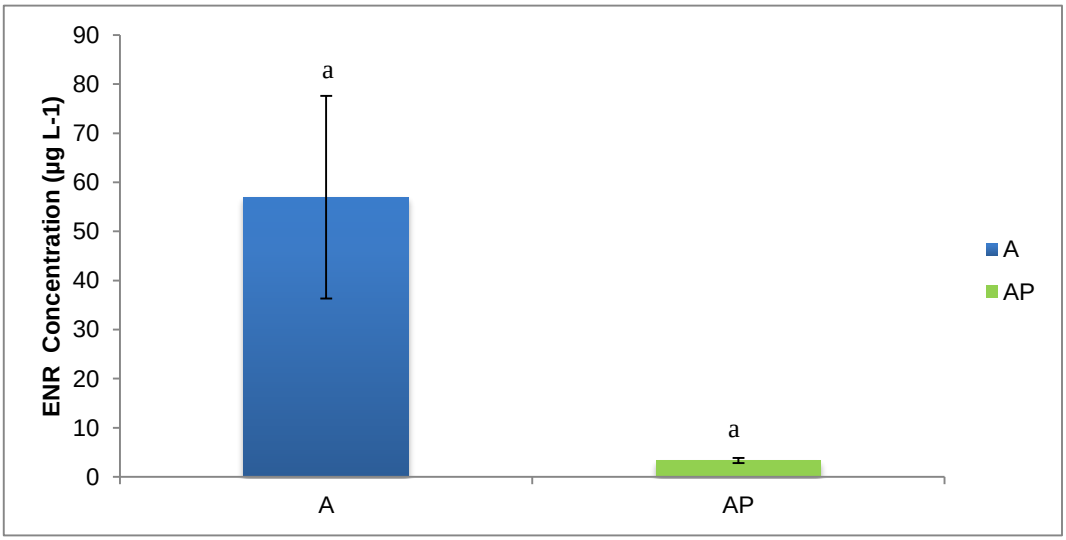


Fig. 29 - ENR concentration in elutriate solution (mean and standard deviation, n=3), after one week exposure to ENR either in the absence (A) or in the presence (AP) of plants. a - significant differences comparing planted and unplanted systems ($p < 0.05$).

Regarding the treatments with elutriate and sediment, a significant reduction of ENR concentration in solution was observed in all treatments after one week of experiment. In fig. 30, the ENR concentrations of elutriates for all treatments at the end of experiment is represented. It was observed that, on average, 95% of ENR was removed from solution comparing with the initial doped concentration (200 µg L^{-1}). Comparing planted and unplanted systems, generally it was observed a significantly higher ENR concentration in unplanted systems ($p < 0.05$). An exception was observed for ENR (E) treatment. It was also observed a significantly higher ENR concentration in the unplanted systems with nutrients (EN S) and with nutrients and glucose (ENC S) when compared with the ENR only, ($p < 0.05$).

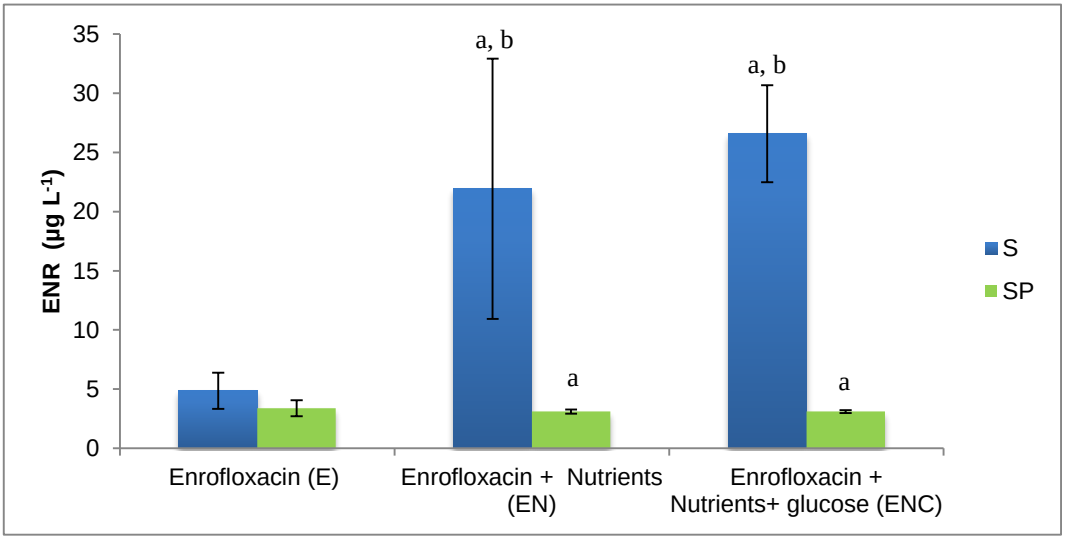


Fig. 30 - ENR concentration in elutriate solution (mean and standard deviation, n=3), after one week with different treatments either in the absence (S) or in the presence (SP) of plants. a - Significant differences comparing planted and unplanted systems ($p < 0.05$). b - Significant differences comparing with ENR treatment (E) ($p < 0.05$).

3.3.2.2. In sediment

Regarding ENR levels in sediments, values below detection limit were obtained for all treatments, therefore it was not possible to identify significant differences between treatments or between unplanted and planted systems.

3.3.3.Microbial abundance

Total cell counts (TCC) was estimated in sediment collected in the sampling site (initial sediment) and in sediment from each treatment after one week of experiment, ranging from 10^6 to10^7 $\log_{10}$ g^{-1} wet sediment (fig. 31). No significant differences were observed ($p > 0.05$) between planted and unplanted systems, or when comparing each treatment with the respective control.

In generally, lower concentrations were observed for all treatments comparing with the initial sediment.

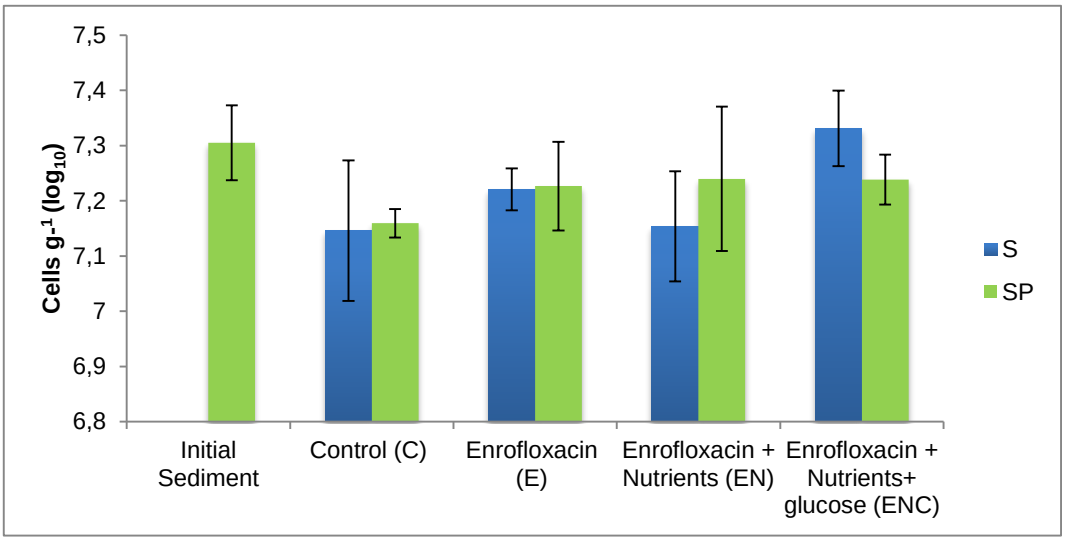


Fig. 31 - - Microbial abundance in sediment (mean and standard deviation, n=3) estimated by DAPI in sediment collected in the sampling site and in sediments, after one week with different treatments either in the absence (S) or in the presence (SP) of plants.

3.3.4.Bacterial richness and diversity

For each treatment, and for the initial sediment, bacterial richness and diversity indexes were calculated from ARISA profiles.

Regarding bacterial richness (fig. 32), estimated by the number of OTUs (ARISA AFLs), generally it was observed no significant effects ($p > 0.05$) comparing unplanted treatments with the respective control. For the unplanted treatments, significant differences ($p < 0.05$) were observed between ENR and ENR with nutrients and glucose and the respective control, the late presenting higher values.

Comparing planted and unplanted systems, in general no significant differences were observed ($p > 0.05$) being the exception the control treatments ($p < 0.05$).

Regarding the initial sediment, in generally, lower concentrations were observed for all treatments.

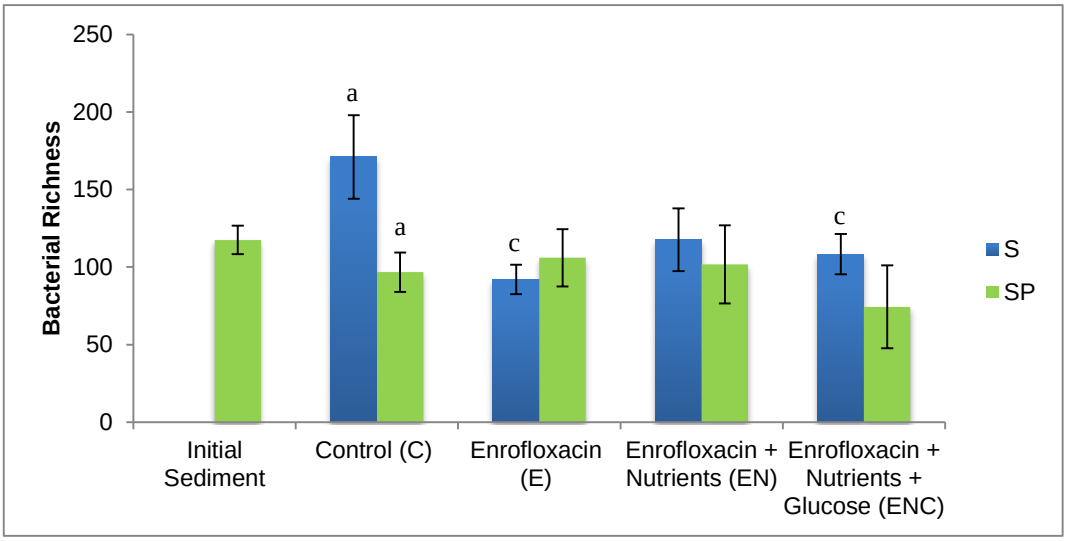


Fig. 32 - Bacterial richness in sediment (mean and standard deviation, n=3) based on ARISA profiles, after one week exposure with different treatments either in the absence (S) or in the presence (SP) of plants. a - significant differences comparing planted and unplanted systems ($p < 0.05$), c - significant differences comparing with respective control ($p < 0.05$).

Regarding diversity index, results presented in fig.33 showed no significant differences ($p > 0.05$) among planted and unplanted systems. Difference was once again observed l for planted and unplanted control.

Comparing each treatment with the respective control, no significant differences ($p > 0.05$) were observed for planted systems. However, significant differences ($p < 0.05$) were observed between all unplanted systems and the respective control (fig. 33).

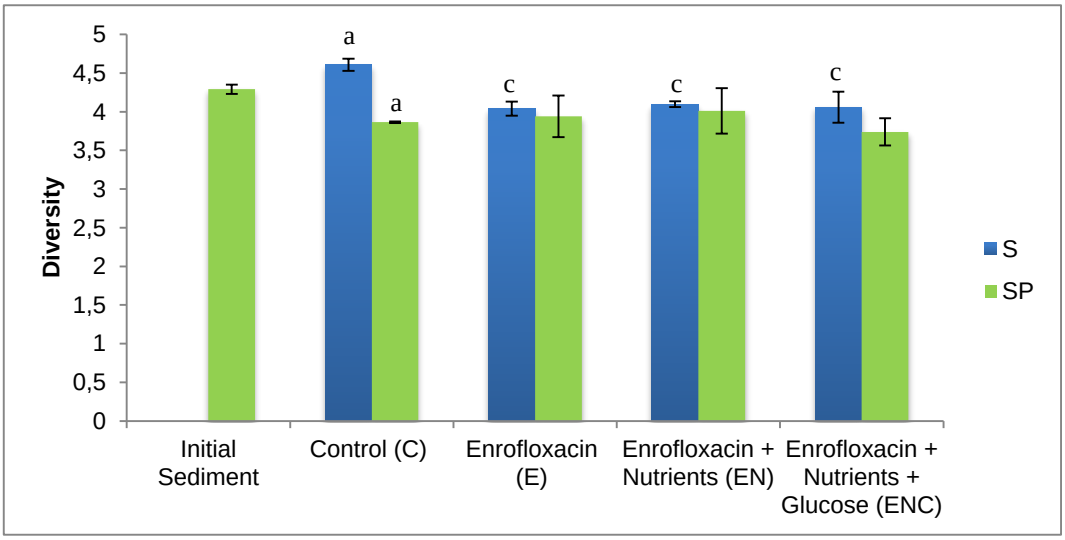


Fig. 33 - Bacterial diversity in sediment (mean and standard deviation, n=3) based on ARISA profiles, after one week with the different treatments either in the absence (S) or in the presence (SP) of plants. a - significant differences comparing planted and unplanted systems ($p < 0.05$); c - significant differences comparing with respective control ($p < 0.05$).

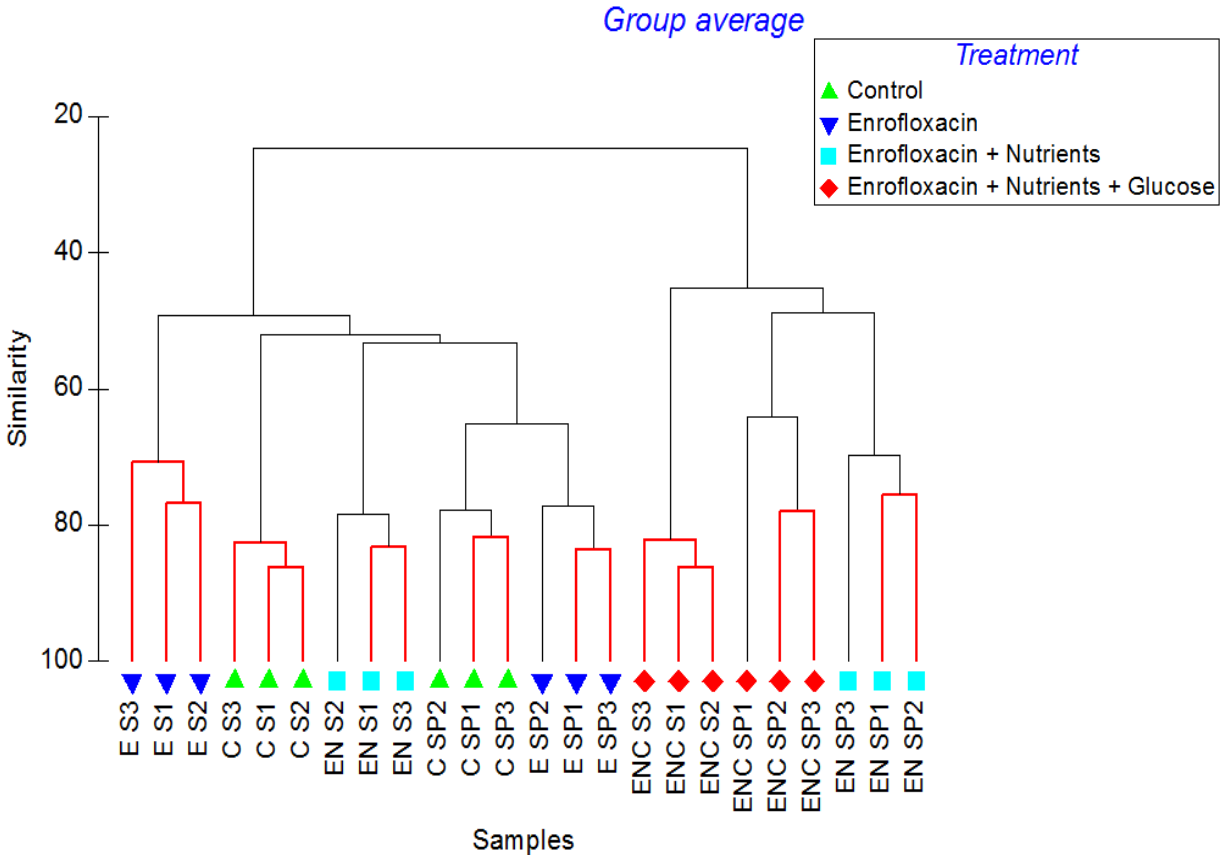
3.3.5. Bacterial community structure

ARISA analysis was performed in sediment collected in the sampling site (initial sediment) and sediment from each treatment (3 replicates per each treatment) at the end of the experiment to characterize the microbial community and try to evaluate the effects of ENR in their community, under different nutritional conditions and in the presence and absence of plant. For each sample, ARISA fragments lengths (ALF) profiles were obtained. The fragments correspond to total number of peaks and therefore to different bacteria phylotypes. Differences in their genetic structure, or more specifically, the distribution of the different phylotypes among the different samples are the most important feature.

A clustering of the samples (fig. 34 - A) with the SIMPROF test (significant differences between samples) was made based on Bray Curtis similarities between samples, in order to evaluate the changes in the microbial community structure. For each treatment, replicates were clustered together, being more similar between each other than with any other sample showing a good experimental replication.

Both cluster analysis and MDS ordination (fig. 34- B) allowed the division of the samples in two main groups with less than 30% of similarity between each other. One of the groups was formed by all the controls and ENR treatments (planted and unplanted) plus the unplanted ENR treatment with nutrients. The other group was formed by the ENR treatments with nutrients and glucose (planted and unplanted) plus the planted ENR treatment with nutrients.

In order to understand the factors responsible for the shaping of the microbial community structure, analysis of similarities (two-way crossed ANOSIM) was performed. Results showed a significant effect of both the presence of plant and type of treatment (table 7) and significant differences between all treatment groups. Therefore, all the variables tested in the experiment (presence/absence of plant, ENR, nutrients and glucose) were relevant for the definition of the microbial community structure.



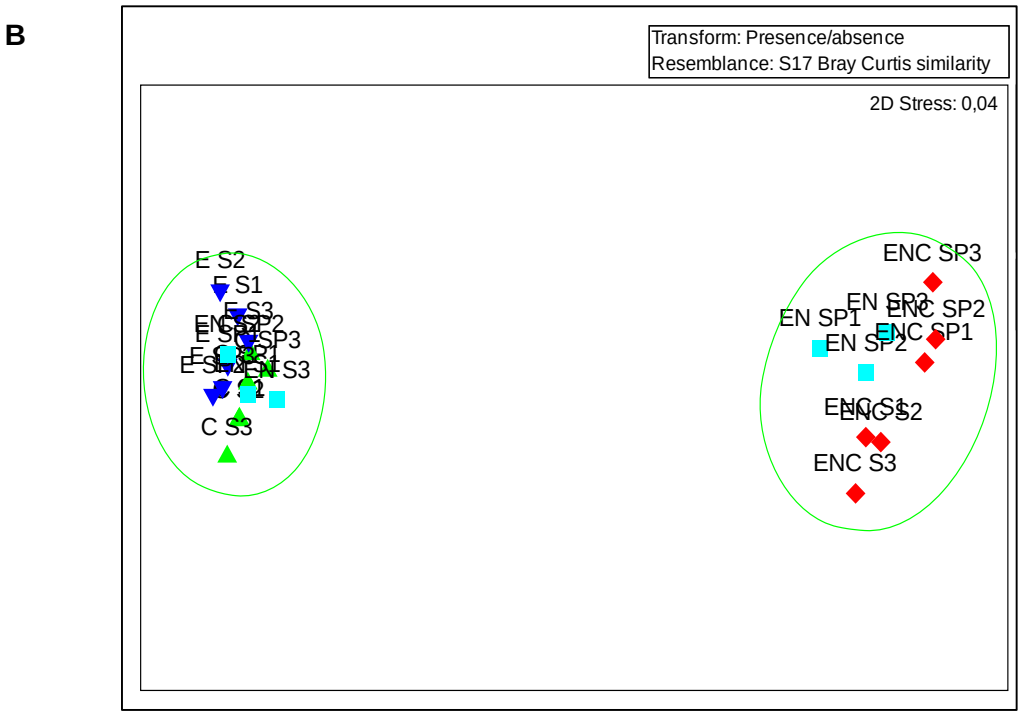


Fig. 34 - Cluster analysis (A) and MDS ordination (B) based on Bray - Curtis similarities of ARISA fingerprints of microbial communities after one week exposure to the different treatments (C – control; E – Enrofloxacin; EN – Enrofloxacin + nutrients; ENC – Enrofloxacin + nutrients + glucose), either in the absence (S) or in the presence (SP) of plants; Points enclosed by the circles cluster at 40% similarity.

Table 7 - Global test of two-way crossed ANOSIM test based on ARISA results after one week exposure to the different treatments (C – control; E – Enrofloxacin; EN – Enrofloxacin + nutrients; ENC – Enrofloxacin + nutrients + glucose), either in the absence (S) or in the presence (SP) of plants.

Differences between:	Statistic value (R)	Significance level (%)
Plant		
Global Test	1	0.1
Treatment		
Global Test	0.978	0.1
Pairwise Tests		
Control, Enrofloxacin	1	1
Enrofloxacin, Enrofloxacin + Nutrients	1	1
Enrofloxacin, Enrofloxacin + Nutrients + Glucose	1	1

3.4. Discussion

In the last few years, estuaries have presented levels of antibiotics contamination including veterinary drugs (e.g. Zheng et al., 2011). In this way, more studies about antibiotics impact in estuarine ecosystems are necessary once they are among the most sensitive ecosystems in the world. It is also important to understand the potential of autochthonous plants and associated microorganisms for the removal of antibiotics from estuarine environment.

Present study was focused in the response of a salt marsh plant-microorganisms association to a contamination with ENR, a veterinary antibiotic, both in terms of ENR removal and changes in the microbial community. Experiments were carried out in the laboratory, in elutriates prepared with estuarine water and sediment. Systems were doped with ENR under different nutritional conditions (with or without extra sources of nutrients and carbon), both in the presence and in the absence of a salt marsh plant (*P. australis*) collected from the same estuary.

At the end of the experiment, systems with nutrients (with or without glucose) were in the best condition, while planted systems without nutrients went into decomposition. The extra source of nutrients appear to be essential for plant maintenance and survival, keeping the systems in good operating conditions.

Regarding elutriate solution, important differences were observed between planted and unplanted systems. Planted systems showed, in general, significantly lower ENR concentrations, demonstrating plant effect in the removal of ENR from solution. In order to isolate the effect of plant from those of sediment and associated microorganisms, additional systems were prepared using filtered elutriate doped with ENR. In these systems the effect of plant was even more notorious as ENR concentration in the planted system were one order of magnitude lower than in unplanted systems. This results are in accordance with what was previously described by Carvalho et al. (2012), using different media (wastewater). The diffusion process of antibiotics into the plant depends on their concentration, water solubility and hydrophobicity (expressed by log K_{ow}) (Dordio & Carvalho, 2013). Moderate hydrophobicity, characterized by log K_{ow} in range of 0.5 to 3.5, is considerate ideal to allow the organic compounds to travel between the lipidic and aqueous phases without getting detained in any of them. ENR presents a moderate hydrophobicity (log K_{ow} of 2.53) and it is soluble enough to move into the cells fluids of the plants demonstrating that the plant can play an important role in the removal of ENR. Nevertheless, ENR may also have caused stress on the plant as reported by Carvalho et al. (2012).

Comparing different treatments, it was observed that for unplanted systems, the presence of nutrients (with or without glucose) inhibit the removal of ENR from solution but that effect was not observed for planted systems. The addition of nutrients in order to stimulate the biodegradation of organic contaminants is a common practice in bioremediation studies (e.g. Almeida et al., 2013), nevertheless, in the present study, the opposite effect was observed. This is in agreements with other authors results (Thiele – Bruhn & Aust, 2004) reporting that stimulating effect of nutrients can be negatively affected by the presence of antibiotics, changing their mobility and availability.

In the case of glucose, added as a source of carbon to benefit co-metabolism, the expected increase in ENR removal was not observed. On the contrary, an inhibition of ENR removal was observed that can be explained by a preference of microorganisms for this more easily degradable source of carbon (Bhatti et al., 2002).

In addition it is necessary to take into account that suspended ENR could have been higher once ENR can aggregate to colloidal matter. Consequentially, a fraction of ENR can be removed during the sample filtration before SPE procedure. During SPE procedure, also occurred ENR losses (recoveries of all procedure of 29%). Therefore, only soluble ENR was measured. Yang et al., (2011) reported that aquatic colloids have a relatively high affinity with pharmaceuticals and colloids can act as strong sorbents.

Enrofloxacin concentration was also measured in sediment samples to evaluate ENR associated with this matrix. Fluoroquinolones, like enrofloxacin, presented strong sorption to soils and sediments, particularly clays (Córdova-Kreylos & Scow, 2007). In the present study, only trace-levels of ENR were detected in sediment matrix being, in most cases, near the detection limit of the method. Slightly higher concentrations of ENR were observed for treatments with glucose, fact that may be related with an inhibition of ENR removal due to the presence of a more bioavailable source of carbon.

Response of microbial community to ENR was evaluated in terms of total abundance, bacterial diversity indexes and bacterial community structure. This was studied in the presence and in the absence of plants and under different nutritional conditions.

In general, no significant differences were observed in terms of total microbial abundance among different treatments or between planted and unplanted systems.

Regarding bacterial diversity indexes, significant differences between plant and unplanted systems were observed only for the control treatment, with higher diversity and richness in the unplanted systems. In general, in unplanted systems it was observed significant lower value of diversity indexes in all treatments with ENR when compared with the control. Therefore, the presence of ENR interfered with the bacterial community, independently of the nutritional conditions, but only in unplanted treatments. Vaclavik et

al. (2004) reported that antibiotics can change bacterial diversity which can indirectly affect soil fertility and nutrient balances. In other study, it was reported a significant diversity decreased when oxytetracycline concentration increased up to 43 mM (Kong et al., 2006).

Shifts on bacterial community structure were analyzed by ARISA. The multivariate analysis of all generated ARISA profiles allowed detection of several differences in terms of community structure between treatments and between planted and unplanted systems. Planted systems were divided in two groups, one formed by the control and ENR treatments and the other formed by the ENR treatments with addition of nutrients alone or with glucose. This separation was in accordance with what was visually observed at the end of the experiment as plants exposed to nutritional supplement were in a better condition, fact that appears to have an effect in its associated microbial community. Unplanted system had a similar separation, except for the unplanted ENR treatment with nutrients that grouped with the control and ENR treatments.

Analysis of similarity showed statistically significant effect of both the presence of plant and type of treatment on the microbial community structure, and significant differences between all treatment groups.

Therefore, the presence or absence of plants was one of the main factors responsible for the shaping of the microbial community structure. Other authors had shown that plants can exert an important influence in their associated microorganisms and can shape microbial communities structure and composition (e.g. Ribeiro et al., 2013). Marschner et al., (2004) reported that plant has a strong influence on the microbial populations around their roots. These shifts in microbial community dynamics, caused by plant, can be related with plant exudation and the interaction of exudates with rhizosphere (Bais et al., 2006). Plants can also promote rhizosphere microbial populations enabling the uptake of limited soil resources (Hamilton & Frank, 2001).

The other relevant factor for microbial community structure definition was the type of treatment, i.e, the presence or absence of ENR, nutrients and glucose. It was already reported the effects of veterinary antibiotics in the structure and functioning of microbial communities. Thiele-Bruhn & Beck, (2005) reported the effects of sulfapyridine and oxytetracycline on soil microbial community in the form of a shift from a bacteria dominated community to a fungi dominated community. In this study, the authors also used glucose in their systems and the observed effects were dependent on the addition of glucose. In other study, it was demonstrated ciprofloxacin capacity to modify microbial community composition at concentrations as low as 20 mg mL^{-1} in anaerobic sediments (Córdova-Kreylos & Scow, 2007).

Soil microbial community can play key roles in ecosystems and influence large number of important ecosystem processes, including nutrient acquisition (Van Der Heijden et al., 2008). Beyond that, soil microbial community is affected by nutrients once they can stimulate their growth and activities (Hammesfahr et al., 2008). The availability of nutrients is known to condition not only biodegradation processes but also the microbial communities involved in biogeochemical processes. Regarding estuarine sediments, Magalhães et al. (2005) showed that inorganic nitrogen concentrations clearly affected the relative abundance of denitrifying/nitrifying bacteria.

With the addition of glucose, an easy degradable source of carbon, important changes in the dominant microbial groups were expected due to an alteration in subtract availability (Bhatti et al., 2002, Thiele-Bruhn & Beck, 2005).

Present study points to the potential of salt-marsh plant-microorganisms association for the bioremediation of antibiotics, despite the specific role of microorganisms in the removal of ENR is still unclear. The degradation of pharmaceuticals by microorganisms is generally slower due to the lack of degradation genes in microorganisms. Nevertheless, some non-specific enzymes can help in the degradation of these compounds (Li et al., 2014).

3.5. Conclusions

The response of a salt marsh plant-microorganisms association to a contamination with a veterinary antibiotic (enrofloxacin) under different nutritional conditions was evaluated using natural estuarine water and sediments. In general, no significant changes were observed in microbial abundance, while the changes in bacterial richness and diversity were observed only in unplanted systems. However, multivariate analysis of ARISA profiles showed significant effect of both the presence of plant and type of treatment on the microbial community structure, and significant differences between all treatment groups. In addition, it was observed that both plants and associated microorganisms present a potential for antibiotic removal that is highly dependent on their nutritional status.

Therefore, the presence of veterinary antibiotics in estuarine areas can affected their microbial communities, influencing their ecosystem and consequently their ecological functions. However, salt marsh plant-microorganisms association has natural potential to attenuate antibiotics contamination and their effects in estuarine areas.

Chapter 4

General Discussion and Conclusions

4. General Discussion and Conclusions

4.1. General discussion

Intensive use of veterinary antibiotics and their continuous input in the environment led to their detection in aquatic matrixes and soil. (Li & Zang, 2010; Zhang et al., 2014). Despite of being found at low concentrations, they can cause toxic effects in organisms and promote antibiotic resistance. Therefore, is crucial to understand the impacts that veterinary antibiotics have in the environment.

Present study was focused on impacts in microbial dynamics and plant interaction in natural wetlands and constructed wetlands, caused by the presence of veterinary antibiotics. It was also studied the potential of both systems to remediate this type of compounds.

Two experiments were carried out, both based on interactions that occur in wetlands; however many factors differentiate one from another. In case of CW's, the wastewater present was coming from a livestock industry. This type of wastewater presents high levels of toxicity and organic matter and the wastewater complexity in terms of pollutants is extremely high which can cause some adverse effects in CW's system. In the other hand, the estuarine water presents other type of complexity: the compounds therein exhibit a high variety and estuarine waters present a salinity gradient that can interfere with the contaminants. Therefore, these two experiments show two different perspectives with the same baseline.

Constructed wetlands have been used in wastewater treatment all over the world. The application of this technology has been extended to livestock wastewater. In a previous work, final removal efficiencies of 98 % for ENR and 94 % for TET were obtained presenting significant reduction in drug concentration (Carvalho et al., 2013).

It was already reported the removing of three veterinary drugs, monensin, narasin and salinomycin, from the wastewater through HSSF-CW (Hussain et al., 2011). In that study, it was obtained removal efficiencies of approximately 40% for monensin and 50 % for narasin and salinomycin. The removal of the same drugs in SF- CW allowed observation of removal efficiencies lower than 40 % (Hussain et al., 2012). The VSSF-CW used in the present study showed higher removal efficiencies comparing with other studies; however the veterinary drugs and CWs configuration were different and the comparison between different types of CWs is not viable (Li et al., 2014) so more studies in this area are necessary.

Liu et al. (2013) reported the removal of ciprofloxacin (fluoroquinolone), oxytetracycline (tetracycline) and sulfamethazine (sulfonamide) from VSSF- CW.

Removals of 91 to 95 % for oxytetracycline, 82 to 85 % to ciprofloxacin and 68 to 63 % for sulfamethazine were obtained. Two of these antibiotics, ciprofloxacin and oxytetracycline, belong to the family of ENR and TET and the type of CW used in present study and in that from Lui et al. (2013) was the same, confirming the efficiency of this type of CW to remove antibiotics and veterinary drugs.

Other types of constructed wetlands also present high removal rates. It has already been reported removal efficiencies higher than 99 % for sulfamethazine (one of the antibiotics tested by Liu et al. (2013) and 98 to 99 % for sulfadiazine in a SF- CW. In that study, it was also reported the successful removal of nutrients (N, P) and COD (84%, 90.4 % and 83.4 % respectively) (Xian et al., 2010). These results emphasize the strong potential of CWs to remove antibiotics from the wastewater keeping its depuration ability to remove other pollutants.

Estuarine ecosystems are among the most productive but also the most sensitive to contamination (Hewson, & Fuhrman, 2004; Mucha et al., 2011) being extremely important their preservation. They have unique physical conditions that support extremely diverse organisms and offer essential relations to near ecosystems (Sun et al., 2012). The presence of chemical contamination, like veterinary antibiotics, implies changes in ecosystem functioning.

In the experiment performed with estuarine conditions, it was also obtained high removals of ENR from water, around 95%. It was observed the potential of natural attenuation of estuaries against ENR. Nevertheless, the input of antibiotics in the environment is continuous and the estuary response can be different. In this experiment, only two doping were carried out during one week and may not translate the real and continuous input of antibiotics in the aquatic media. Furthermore, the presence of plant seems to affect the removal efficiency of ENR, which was not clearly observed in CW's microcosms. High removal efficiencies were obtained for planted systems (around 98%) comparing with unplanted systems (approximately 91%).

The presence of glucose and nutrients had a negative effect in ENR removal efficiency obtained, being more pronounced in unplanted systems. Nutrients stimulation may have been inhibited by the presence of ENR, affecting their mobility and availability (Thiele – Bruhn & Aust, 2004). In the other hand, glucose is an easily degradable substrate (Bhatti et al., 2002) comparing with ENR leading to its degradation instead antibiotic degradation. Normally, the addition of glucose leads to a faster growth rate and more biomass yield (Bhatti et al., 2002), stimulating the organic compounds degradation.

The input of nutrients in estuarine areas through rivers, lagoons, soil lixiviation, treated wastewater or by diffused sources has increased along the years leading to estuaries eutrophication (Bouvy et al., 2010). If the initiatory effect, which was observed

in this study, occurs in estuaries, the natural degradation of veterinary antibiotics can be compromised and their accumulation in estuarine ecosystems will increase.

In addition, ENR treatment with nutrients and glucose (planted and unplanted) can be compared with the constructed wetland simulated systems once the wastewater also contains organic matter and nutrients in their composition. However, in CW, it was not clear the inhibitory effect of nutrients and organic matter (extra source of carbon and, in general, much easier to degrade) in the removal of antibiotics as well in the depuration ability. In this way, more studies are needed to understand the effect of nutrients and extra sources of carbon in the removal of antibiotics.

Comparing the two studied systems, some similarities were observed in terms of microbial community response as well in microbial abundance and diversity.

Shifts in microbial community structure due to plant and type of treatment were observed in both studies for ENR. Nevertheless, in constructed wetlands microcosm, the most determinant factor was type of treatment and the plant effect was not as evident as that obtained for estuarine waters. The greater complexity and toxicity of wastewater as compared with estuarine waters leads to the need of plant adaptation to different conditions which may be related to the observed difference. Plant, in both cases, was an important factor in the microbial community definition. Plant exudates can be related with this (Bais et al., 2006, Koranda et al., 2011) as well as some plant stress caused by antibiotics (Carvalho et al., 2012).

Treatment type also had a significant impact on microbial community structure. Changes in microbial community structure due to antibiotics have been reported by several authors (Reichel et al., (2013); Hammesfahr et al., (2008); Thiele-Bruhn &Beck, 2005).

In both studies, no significant differences were observed in microbial abundance. In case of diversity, for constructed wetlands, no significant effects were observed for each planted and unplanted treatment; however, for estuarine waters, bacterial diversity was affected by ENR in all unplanted systems. Different effect was observed for diversity in constructed wetlands. In this case, for week 2 and 4, in unplanted systems, was observed higher diversity for wastewater doped with ENR. Variation in unplanted treatments does not present a clear pattern, being necessary more studies to evaluate fluctuations in bacterial diversity due ENR exposure. However, changes in bacterial diversity caused by veterinary antibiotics exposure have been previously reported (Vaclavik et al., 2004; Kong et al., 2006; Ollivier et al., 2013; Jechalke et al., 2014).

When comparing control with tetracycline treatment in planted CWs systems, it was observed that, time was the most important factor defining community structure, followed by treatment type. The results showed an adaptation process of microbial

community. Adaptation processes can't be compared among studies. In the experiment performed with estuarine conditions, time of exposure was only one week being this time not enough to observe the adaptation of communities.

Veterinary pharmaceuticals are known to cause changes in microbial community affecting, this way, ecological functions of soil ecosystems (Thiele-Bruhn & Beck 2005; Kotzerke et al., 2008). Nevertheless, toxicity test performed in the CWs systems used in present study (Carvalho 2013) showed that they were able to decrease wastewater toxicity, independently of the antibiotic presence maintaining the depuration ability.

Therefore, the exposure to veterinary drugs leads to significant changes in microbial community structure; nevertheless, wetlands had the potential to remove veterinary antibiotics from water and maintain depuration ability.

4.2. Conclusion

Both experiments pointed to the potential of wetlands, both natural wetlands (estuaries) and constructed wetlands to attenuate antibiotics contamination.

In general, no significant differences were observed in terms of microbial abundance among different treatments. Nonetheless, multivariate analysis of ARISA profiles showed several differences in terms of community structure among treatments.

In case of CW's time of exposure was the most important factor in defining bacterial community structure, followed by the type of treatment; however plants presence explained part of the observed differences. In the other hand, in the experiment performed with natural wetlands conditions, plant was the most important factor defining community structure followed by type of treatment, confirming, in certain way, the results obtained in the CW's experiment.

This study emphasizes the importance of natural wetlands in terms of bioremediation potential. Constructed wetlands present a potential and sustainable alternative to remove veterinary antibiotics from livestock wastewater without significant changes in bacterial diversity and maintaining their depuration ability. CW is a promising technology, based on natural wetlands, to remediate environmental impact caused by livestock industry. The interaction between microorganisms and plant seems to be the most important removal mechanism presented in these systems, being the time also very important in the adaptation process of microbial community.

Chapter 5

References

5. References

Allison, S. D., & Martiny, J. B. (2008). Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences, 105(Supplement 1), 11512-11519.

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

Almeida C.M, Reis I., Couto N., Bordalo A.A., Mucha A.P. (2013). Potential of the microbial community present in an un-impacted beach sediment to remediate petroleum hydrocarbons. Environmental Science and Pollution Research, 20: 3176-3184.

Armstrong, J., & Armstrong, W. (1988). Phragmites australis–A preliminary study of soil-oxidizing sites and internal gas transport pathways. New Phytologist, 108(4), 373-382.

Babić, S., Pavlović, D. M., Ašperger, D., Periša, M., Zrnčić, M., Horvat, A. J., Kaštelan-Macan, M. (2010). Determination of multi-class pharmaceuticals in wastewater by liquid chromatography–tandem mass spectrometry (LC–MS–MS). Analytical and Bioanalytical Chemistry, 398(3), 1185-1194.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol., 57, 233-266.

Barrett, J. E., Virginia, R. A., Wall, D. H., Cary, S. C., Adams, B. J., Hacker, A. L., Aislabie, J. M. (2006). Co-variation in soil biodiversity and biogeochemistry in northern and southern Victoria Land, Antarctica. Antarctic Science, 18(04), 535-548.

Berglund, B., Khan, G. A., Weisner, S. E., Ehde, P. M., Fick, J., Lindgren, P. E. (2014). Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. Science of the Total Environment, 476, 29-37.

Bhatti, Z. I., Toda, H., Furukawa, K. (2002). *p-Nitrophenol* degradation by activated sludge attached on nonwovens. Water Research, 36(5), 1135-1142.

Bissett, A., Burke, C., Cook, P. L., Bowman, J. P. (2007). Bacterial community shifts in organically perturbed sediments. Environmental microbiology, 9(1), 46-60.

Bouvy, M., Arfi, R., Bernard, C., Carré, C., Got, P., Pagano, M., Troussellier, M. (2010). Estuarine microbial community characteristics as indicators of human-induced changes (Senegal River, West Africa). Estuarine, Coastal and Shelf Science, 87(4), 573-582.

Brix, H. (1994). Use of constructed wetlands in water pollution control: historical development, present status, and future perspectives. Water Science and Technology, 30(8), 209-224.

Brusati, E. D., & Grosholz, E. D. (2006). Native and introduced ecosystem engineers produce contrasting effects on estuarine infaunal communities.Biological Invasions, 8(4), 683-695.

Carvalho, P. N., Basto, M.C.P, Almeida, C. M. R. (2012). Potential of Phragmites australis for the removal of veterinary pharmaceuticals from aquatic media. Bioresource Technology, 116, 497-501.

Carvalho, P. N., (2012). Implementation of methodologies for removal of veterinary pharmaceuticals residues from WWTPs effluents of the livestock industry. PhD Thesis in Chemistry. Faculdade de Ciências da Universidade do Porto.

Carvalho, P. N., Araújo, J. L., Mucha, A. P., Basto, M. C. P., Almeida, C. M. R. (2013). Potential of constructed wetlands microcosms for the removal of veterinary pharmaceuticals from livestock wastewater. Bioresource Technology, 134, 412-416

Carvalho, P. N., Pirra, A., Basto, M. C. P., Almeida, C. M. R. (2013b). Multi-family methodologies for the analysis of veterinary pharmaceutical compounds in sediment and sludge samples: comparison among extraction techniques. Analytical Methods, 5, 6503-6510.

Carvalho, P. N., Basto, M. C. P., Almeida, C. M. R., Brix, H. (2014). A review of plant–pharmaceutical interactions: from uptake and effects in crop plants to phytoremediation in constructed wetlands. Environmental Science and Pollution Research, 1-35.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A. M., Rizzi, A., ... Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Applied and Environmental Microbiology, 70(10), 6147-6156.

Cavenati, S., Carvalho, P. N., Almeida, C. M. R., Basto, M.C.P, Vasconcelos, M. T. S. D. (2012). Simultaneous determination of several veterinary pharmaceuticals in effluents from urban, livestock and slaughterhouse wastewater treatment plants using a simple chromatographic method. Water Science & Technology, 66, 603 - 611

Chapman, P. M., & Wang, F. (2001). Assessing sediment contamination in estuaries. Environmental Toxicology and Chemistry, 20(1), 3-22.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews, 65(2), 232-260.

Clarke, K. R., & Gorley, R. N. (2006). PRIMER v6: user manual/tutorial. Plymouth Marine Laboratory, Plymouth.

Córdova-Kreylos, A. L., & Scow, K. M. (2007). Effects of ciprofloxacin on salt marsh sediment microbial communities. The ISME journal, 1(7), 585-595.

Danovaro, R., Luna, G. M., Dell'Anno, A., Pietrangeli, B. (2006). Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. Applied and Environmental Microbiology, 72(9), 5982-5989.

Danovaro, R., Corinaldesi, C., Luna, G. M., Magagnini, M., Manini, E., Pusceddu, A. (2009). Prokaryote diversity and viral production in deep-sea sediments and seamounts. Deep Sea Research Part II: Topical Studies in Oceanography, 56(11), 738-747.

DGAV: Direção Nacional de Alimentação e Veterinária. Available in: http://www.dgv.min-agricultura.pt/portal/page/portal/DGV

Deng, Y., Zhang, Y., Gao, Y., Li, D., Liu, R., Liu, M., ... Yang, M. (2011). Microbial community compositional analysis for series reactors treating high level antibiotic wastewater. Environmental science & technology, 46(2), 795-801.

Ding, C., & He, J. (2010). Effect of antibiotics in the environment on microbial populations. Applied microbiology and biotechnology, 87(3), 925-941.

Dordio, A., Carvalho, A. J. P., Teixeira, D. M., Dias, C. B., Pinto, A. P. (2010). Removal of pharmaceuticals in microcosm constructed wetlands using Typha spp. and LECA. Bioresource Technology 101, 886–892.

Dordio, A. V., & Carvalho, A. J. P. (2013). Organic xenobiotics removal in constructed wetlands, with emphasis on the importance of the support matrix. Journal of Hazardous materials, 252, 272-292.

Dugan, P. J. (Ed.). (1990). Wetland conservation: A review of current issues and required action. IUCN.

ECDC (2011): Surveillance of antimicrobial consumption in Europe, 2011. Available in: http://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-consumption-europe-surveillance-2011.pdf

Eggleton, J., & Thomas, K. V. (2004). A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events.Environment international, 30(7), 973-980.

Euroactiv (2013): New chemicals, drugs added to EU water pollution watch list. Available in: http://www.euractiv.com/health/new-chemicals-pharmaceuticals-ad-news-529073. Last accessed in 24/09/2014.

Fatta-Kassinos, D., Kalavrouziotis, I. K., Koukoulakis, P. H., Vasquez, M. I. (2011). The risks associated with wastewater reuse and xenobiotics in the agroecological environment. Science of the Total Environment, 409(19), 3555-3563.

Fent, K., Escher, C., Caminada, D. (2006). Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reproductive Toxicology, 22(2), 175-185.

Fester, T., Giebler, J., Wick, L. Y., Schlosser, D., Kästner, M. (2014). Plant–microbe interactions as drivers of ecosystem functions relevant for the biodegradation of organic contaminants. Current opinion in biotechnology, 27, 168-175.

Fisher, M. M., & Triplett, E. W. (1999). Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Applied and Environmental Microbiology, 65(10), 4630-4636.

Garcia, J., Rousseau, D. P., Morato, J., Lesage, E. L. S., Matamoros, V., Bayona, J. M. (2010). Contaminant removal processes in subsurface-flow constructed wetlands: a review. Critical Reviews in Environmental Science and Technology, 40(7), 561-661.

Garcia-Rodríguez, A., Matamoros, V., Fontàs, C., Salvadó, V. (2014). The ability of biologically based wastewater treatment systems to remove emerging organic contaminants—a review. Environmental Science and Pollution Research. DOI 10.1007/ s11356-013-2448-5

Gavrilescu, M., Demnerová, K., Aamand, J., Agathos, S., Fava, F. (2014). Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. New biotechnology. DOI:10.1016/j.nbt.2014.01.001

Gillanders, B. M., Elsdon, T. S., Halliday, I. A., Jenkins, G. P., Robins, J. B., Valesini, F. J. (2011). Potential effects of climate change on Australian estuaries and fish utilising estuaries: a review. Marine and Freshwater Research, 62(9), 1115-1131.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhøft, H. C., Jørgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment-A review. Chemosphere, 36(2), 357-393.

Hamilton III, E. W., & Frank, D. A. (2001). Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecology, 82(9), 2397-2402.

Hammesfahr, U., Heuer, H., Manzke, B., Smalla, K.,Thiele-Bruhn, S. (2008). Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. Soil Biology and Biochemistry, 40(7), 1583-1591.

Hammesfahr, U., Kotzerke, A., Lamshöft, M., Wilke, B. M., Kandeler, E., Thiele-Bruhn, S. (2011). Effects of sulfadiazine-contaminated fresh and stored manure on a soil microbial community. European Journal of Soil Biology, 47(1), 61-68.

Helt, C. D., Weber, K. P., Legge, R. L., Slawson, R. M. (2012). Antibiotic resistance profiles of representative wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms. Ecological Engineering, 39, 113-122.

Hewson, I., & Fuhrman, J. A. (2004). Richness and diversity of bacterioplankton species along an estuarine gradient in Moreton Bay, Australia. Applied and Environmental Microbiology, 70(6), 3425-3433.

Hussain, S. A., Prasher, S. O., Patel, R. (2011). Removal efficiency of horizontal subsurface flow wetlands for veterinary pharmaceuticals. Transactions of the ASABE, 54(6), 2037-2046.

Hussain, S. A., Prasher, S. O., Patel, R. M. (2012). Removal of ionophoric antibiotics in free water surface constructed wetlands. Ecological engineering, 41, 13-21.

Ingerslev, F., Torång, L., Loke, M. L., Halling-Sørensen, B., Nyholm, N. (2001). Primary biodegradation of veterinary antibiotics in aerobic and anaerobic surface water simulation systems. Chemosphere, 44(4), 865-872.

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K. (2014). Fate and effects of veterinary antibiotics in soil. Trends in Microbiology. DOI 10.1016/j.tim.2014.05.005

Jones, O. A. H., Voulvoulis, N., Lester, J. N. (2001). Human pharmaceuticals in the aquatic environment a review. Environmental technology, 22(12), 1383-1394.

Jones, H. F., Pilditch, C. A., Bruesewitz, D. A., Lohrer, A. M. (2011). Sedimentary environment influences the effect of an infaunal suspension feeding bivalve on estuarine ecosystem function. PloS one, 6(10), e27065.

Johnson, D. L., Maguire, K. L., Anderson, D. R., McGrath, S. P. (2004). Enhanced dissipation of chrysene in planted soil: the impact of a rhizobial inoculum. Soil Biology and Biochemistry, 36(1), 33-38.

JRC Reports (2012): Science for Water - JRC thematic report, 2012. Available in: http://ec.europa.eu/dgs/jrc/downloads/jrc_2012_science_for_water_en.pdf

Kadlec, R. H., & Wallace, S. (2008). Treatment wetlands. CRC press.

Kepner, R. L., & Pratt, J. R. (1994). Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiological Reviews, 58(4), 603-615.

Kim, S., & Aga, D. S. (2007). Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. Journal of Toxicology and Environmental Health, Part B, 10(8), 559-573.

Kim, Y., Jung, J., Kim, M., Park, J., Boxall, A., Choi, K. (2008). Prioritizing veterinary pharmaceuticals for aquatic environment in Korea. Environmental toxicology and pharmacology, 26(2), 167-176.

Kivaisi, A. K. (2001). The potential for constructed wetlands for wastewater treatment and reuse in developing countries: a review. Ecological Engineering, 16(4), 545-560.

Kotzerke, A., Sharma, S., Schauss, K., Heuer, H., Thiele-Bruhn, S., Smalla, K., Schloter, M. (2008). Alterations in soil microbial activity and< i> N</i>-transformation processes due to sulfadiazine loads in pig-manure. Environmental Pollution, 153(2), 315-322.

Klosterhaus, S. L., Grace, R., Hamilton, M. C., Yee, D. (2013). Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in surface waters, sediments, and mussels in an urban estuary. Environment international, 54, 92-99.

Knapp, C. W., Cardoza, L. A., Hawes, J. N., Wellington, E. M. H., Larive, C. K., Graham, D. W. (2005). Fate and effects of enrofloxacin in aquatic systems under different light conditions. Environmental science & technology, 39(23), 9140-9146.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environmental science & technology, 36(6), 1202-1211.

Kong, W. D., Zhu, Y. G., Fu, B. J., Marschner, P., He, J. Z. (2006). The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community. Environmental Pollution, 143(1), 129-137.

Koranda, M., Schnecker, J., Kaiser, C., Fuchslueger, L., Kitzler, B., Stange, C. F., ... Richter, A. (2011). Microbial processes and community composition in the rhizosphere

of European beech–the influence of plant C exudates. Soil Biology and Biochemistry, 43(3), 551-558.

Kümmerer, K. (2009a). Antibiotics in the aquatic environment–a review–part I. Chemosphere, 75(4), 417-434.

Kümmerer, K. (2009b). Antibiotics in the aquatic environment–a review–part II. Chemosphere, 75(4), 435-441.

Li, B., & Zhang, T. (2010). Biodegradation and adsorption of antibiotics in the activated sludge process. Environmental science & technology, 44(9), 3468-3473.

Li, W. C. (2014). Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. Environmental Pollution, 187, 193-201.

Li, Y., Zhu, G., Ng, W. J.,Tan, S. K. (2014). A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: design, performance and mechanism. Science of The Total Environment, 468, 908-932.

Lapworth, D. J., Baran, N., Stuart, M. E., Ward, R. S. (2012). Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. Environmental Pollution, 163, 287-303.

Lozupone, C. A., & Knight, R. (2007). Global patterns in bacterial diversity. Proceedings of the National Academy of Sciences, 104(27), 11436-11440.

Liu, L., Liu, C., Zheng, J., Huang, X., Wang, Z., Liu, Y., Zhu, G. (2013). Elimination of veterinary antibiotics and antibiotic resistance genes from swine wastewater in the vertical flow constructed wetlands. Chemosphere, 91(8), 1088-1093.

Magalhães, C. M., Joye, S. B., Moreira, R. M., Wiebe, W. J., Bordalo, A. A. (2005). Effect of salinity and inorganic nitrogen concentrations on nitrification and denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, Portugal. *Water Research*, *39*(9), 1783-1794.

Malik, S., Beer, M., Megharaj, M., Naidu, R. (2008). The use of molecular techniques to characterize the microbial communities in contaminated soil and water. Environment International, 34(2), 265-276.

Marschner, P., Crowley, D., Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant and Soil, 261(1-2), 199-208.

Meador, J. P., Casillas, E., Sloan, C. A., Varanasi, U. (1995). Comparative bioaccumulation of polycyclic aromatic hydrocarbons from sediment by two infaunal invertebrates. Marine ecology progress series. Oldendorf, 123(1), 107-124.

Meers, E., Tack, F. M. G., Tolpe, I., Michels, E. (2008). Application of a full-scale constructed wetland for tertiary treatment of piggery manure: monitoring results. Water, Air, and Soil Pollution, 193(1-4), 15-24.

Metcalf, I. N. C., & Eddy, H. (2003). Wastewater engineering: treatment and reuse. McGRAW-HILL.

Milić, N., Milanović, M., Letić, N. G., Sekulić, M. T., Radonić, J., Mihajlović, I., Miloradov, M. V. (2013). Occurrence of antibiotics as emerging contaminant substances in aquatic environment. International journal of environmental health research, 23(4), 296-310.

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

Nunes da Silva, M., Mucha, A. P., Rocha, A. C., Silva, C., Carli, C., Gomes, C. R., Almeida, C. M. R. (2014). Evaluation of the ability of two plants for the phytoremediation of Cd in salt marshes. Estuarine, Coastal and Shelf Science,141, 78-84.

Oliveira, V., Gomes, N. C., Almeida, A., Silva, A. M., Silva, H., Cunha, Â. (2014). Microbe-Assisted Phytoremediation of Hydrocarbons in Estuarine Environments. Microbial ecology, 1-12.

Ollivier, J., Schacht, D., Kindler, R., Groeneweg, J., Engel, M., Wilke, B. M., ... Schloter, M. (2013). Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. Frontiers in microbiology, 4.

Pan, K., & Wang, W. X. (2012). Trace metal contamination in estuarine and coastal environments in China. Science of the total environment, 421, 3-16.

Park, J., Kim, M. H., Choi, K., Kim, Y. H., Kim, M. Y. (2007). Environmental Risk Assessment of Pharmaceuticals: Model Application for Estimating Pharmaceutical Exposures in the Han River Basin. Korea Environment Institute.(KEI 2007 RE-06).

Pei, R., Cha, J., Carlson, K. H., Pruden, A. (2007). Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. Environmental science & technology, 41(14), 5108-5113.

Porter, K. G., & Feig, Y. S. (1980). Use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography. 25, 943-8

Prosser, J. I., Rangel-Castro, J. I., Killham, K. (2006). Studying plant–microbe interactions using stable isotope technologies. Current opinion in biotechnology,17(1), 98-102.

Qiao, M., Chen, W., Su, J., Zhang, B., Zhang, C. (2012). Fate of tetracyclines in swine manure of three selected swine farms in China. Journal of Environmental Sciences, 24(6), 1047-1052.

Ranjard, L., Poly, F., Lata, J. C., Mougel, C., Thioulouse, J., Nazaret, S. (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Applied and Environmental Microbiology, 67(10), 4479-4487.

Reichel, R., Rosendahl, I., Peeters, E. T., Focks, A., Groeneweg, J., Bierl, R., Thiele-Bruhn, S. (2013). Effects of slurry from sulfadiazine-(SDZ) and difloxacin-(DIF) medicated pigs on the structural diversity of microorganisms in bulk and rhizosphere soil. Soil Biology and Biochemistry, 62, 82-91.

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

Ribeiro, H., Mucha, A. P., Marisa R Almeida, C., Bordalo, A. A. (2013). Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh. Science of The Total Environment, 458, 568-576.

Ribeiro H., Mucha A.P., Almeida C.M.R., Bordalo, A.A. (2014). Salt marsh plants potential to remove petroleum hydrocarbon: rhizoremediation, biostimulation or bioaugmentation? Journal of Environmental Management. (http://dx.doi.org/10.1016/j.jenvman.2014.01.047)

Rivera-Utrilla, J., Sánchez-Polo, M., Ferro-García, M. Á., Prados-Joya, G., Ocampo-Pérez, R. (2013). Pharmaceuticals as emerging contaminants and their removal from water. A review. Chemosphere, 93(7), 1268-1287.

Salt, D. E., Smith, R. D., Raskin, I. (1998). Phytoremediation. Annual review of plant biology, 49(1), 643-668.

Salvato, M., Borin, M., Doni, S., Macci, C., Ceccanti, B., Marinari, S., Masciandaro, G. (2012). Wetland plants, micro-organisms and enzymatic activities interrelations in treating N polluted water. Ecological Engineering, 47, 36-43.

Sarmah, A. K., Meyer, M. T., Boxall, A. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere, 65(5), 725-759.

Scholz, M., & Lee, B. H. (2005). Constructed wetlands: a review. International journal of environmental studies, 62(4), 421-447.

Stewart, M., Olsen, G., Hickey, C. W., Ferreira, B., Jelić, A., Petrović, M., Barcelo, D. (2014). A survey of emerging contaminants in the estuarine receiving environment around Auckland, New Zealand. Science of the Total Environment, 468, 202-210.

Stottmeister, U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., ... Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnology Advances, 22(1), 93-117.

Sun, J., Wang, M. H., Ho, Y. S. (2012). A historical review and bibliometric analysis of research on estuary pollution. Marine pollution bulletin, 64(1), 13-21.

Thiele-Bruhn, S., & Aust, M. O. (2004). Effects of pig slurry on the sorption of sulfonamide antibiotics in soil. Archives of environmental contamination and toxicology, 47(1), 31-39.

Thiele-Bruhn, S., & Beck, I. C. (2005). Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere,59(4), 457-465.

Thomaidis, N. S., Asimakopoulos, A. G., Bletsou, A. A. (2012). Emerging contaminants: a tutorial mini-review. Global NEST Journal, 14(1), 72-79.

Thomas, F., Giblin, A. E., Cardon, Z. G., Sievert, S. M. (2014). Rhizosphere heterogeneity shapes abundance and activity of sulfur-oxidizing bacteria in vegetated salt marsh sediments. Terrestrial Microbiology, 5, 309.

Trovó, A. G., Melo, S. A. S., Nogueira, R. F. P. (2008). Photodegradation of the pharmaceuticals amoxicillin, bezafibrate and paracetamol by the photo-Fenton process—application to sewage treatment plant effluent. Journal of Photochemistry and Photobiology A: Chemistry, 198(2), 215-220.

Thuy, H. T. T., & Loan, T. T. C. (2014). Degradation of Selected Pharmaceuticals in Coastal Wetland Water and Sediments. Water, Air, & Soil Pollution, 225(5), 1-9.

Ulitzur, S., Lahav, T., Ulitzur, N. (2002). A novel and sensitive test for rapid determination of water toxicity. Environmental Toxicology, 17(3), 291-296.

USEPA, U. S. Army Corps of Engineers (1991). Evaluation of dredged material proposed for ocean disposal. Washington, DC, USA.

Vaclavik, E., Halling-Sørensen, B., Ingerslev, F. (2004). Evaluation of manometric respiration tests to assess the effects of veterinary antibiotics in soil. Chemosphere, 56(7), 667-676.

Van Der Heijden, M. G., Bardgett, R. D., Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology letters, 11(3), 296-310.

Verlicchi, P., Al Aukidy, M., Zambello, E. (2012). Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—a review. Science of the Total Environment, 429, 123-155.

Verlicchi, P., & Zambello, E. (2014). How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Science of The Total Environment, 470, 1281-1306.

Warwick, R. M., & Clarke, K. R. (1991). A comparison of some methods for analysing changes in benthic community structure. Journal of the Marine Biological Association of the United Kingdom, 71(01), 225-244.

Weis, P., Windham, L., Burke, D. J., Weis, J. S. (2002). Release into the environment of metals by two vascular salt marsh plants. Marine Environmental Research, 54(3), 325-329.

Wille, K., Noppe, H., Verheyden, K., Bussche, J. V., De Wulf, E., Van Caeter, P., ... Vanhaecke, L. (2010). Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. Analytical and bioanalytical chemistry, 397(5), 1797-1808.

Williams, J. B. (2002). Phytoremediation in wetland ecosystems: progress, problems, and potential. Critical Reviews in Plant Sciences, 21(6), 607-635.

Wu, S., Kuschk, P., Brix, H., Vymazal, J., Dong, R. (2014). Development of constructed wetlands in performance intensifications for wastewater treatment: A nitrogen and organic matter targeted review. Water research, 57, 40-55.

Xian, Q., Hu, L., Chen, H., Chang, Z., Zou, H. (2010). Removal of nutrients and veterinary antibiotics from swine wastewater by a constructed macrophyte floating bed system. Journal of environmental management, 91(12), 2657-2661.

Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., Zhou, J. L. (2011). Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. Journal of hazardous materials, 190(1), 588-596.

Zhang, W., Huang, M. H., Qi, F. F., Sun, P. Z., Van Ginkel, S. W. (2013). Effect of trace tetracycline concentrations on the structure of a microbial community and the development of tetracycline resistance genes in sequencing batch reactors. Bioresource Technology, 150, 9-14.

Zhang, D., Gersberg, R. M., Ng, W. J., Tan, S. K. (2014). Removal of pharmaceuticals and personal care products in aquatic plant-based systems: A review. Environmental Pollution, 184, 620-639.

Zhang, D. Q., Jinadasa, K. B. S. N., Gersberg, R. M., Liu, Y., Ng, W. J., Tan, S. K. (2014b). Application of constructed wetlands for wastewater treatment in developing countries–A review of recent developments (2000–2013). Journal of environmental management, 141, 116-131.

Zheng, S., Qiu, X., Chen, B., Yu, X., Liu, Z., Zhong, G., ... Freestone, D. (2011). Antibiotics pollution in Jiulong River estuary: source, distribution and bacterial resistance. Chemosphere, 84(11), 1677-1685.

Zhou, J. L., Zhang, Z. L., Banks, E., Grover, D., Jiang, J. Q. (2009). Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. Journal of Hazardous Materials, 166(2), 655-661.